THE UNIVERSITY OF ALBERTA

IMPORTANCE OF ASPARTATE 131 FOR T4 DNA POLYMERASE PROOFREADING MECHANISM

by

Rosanna Pearl Baker

A THESIS

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Genetics

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled IMPORTANCE OF ASPARTATE 131 FOR T4 DNA POLYMERASE PROOFREADING MECHANISM submitted by Rosanna Pearl Baker in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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John Bell

Brenda Leskiw

Date: Sept 23, 1996

You work that you may keep pace with the earth and the soul of the earth...
When you work you are a flute through whose heart the whispering of the hours turns to music.
...when you work you fulfil a part of earth's dream, assigned to you when that dream was born,
And in keeping yourself with labour you are in truth loving life,
And to love life through labour is to be intimate with life's inmost secret.

Kahlil Gibran, 1923

ABSTRACT

The study of mutant DNA polymerases with decreased 3'→5' exonuclease activity has provided insight into the proofreading mechanism. Three mutant T4 DNA polymerases with different amino acid substitutions for aspartate-131 were identified in genetic selections for mutants with increased spontaneous mutation rates. The D131G-DNA polymerase displays a strong mutator phenotype *in vivo*, while D131N- and D131S-DNA polymerases have weaker mutator phenotypes. The repeated isolation of mutants with amino acid substitutions at D131 suggests that this residue is important for proofreading function. The role of D131 in proofreading was investigated by biochemically characterizing the mutant enzymes, using both standard enzyme assays and a more sensitive fluorescence-based assay that measures excision rates on a millisecond time scale. The results, combined with recent crystallographic studies, suggest that D131 contributes to the structural integrity of the T4 DNA polymerase exonuclease active center and is, for this reason, vital to proofreading function.

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TABLE OF CONTENTS

INTRODUCTION 1

MATERIALS AND METHODS 14

Microbiological Media 14

Growth of Bacteria and Bacteriophage 14

Construction of T4 DNA Polymerase Mutants 16

Confirmation of T4 DNA Polymerase Mutations 18

Purification of T4 DNA Polymerase Mutant Enzymes 21

Transfer of Constructed Mutations into Bacteriophage T4 by Homologous Recombination 23

Characterization of T4 DNA Polymerase Mutants 25

T4 DNA Polymerase 5'→3' Polymerase Activity Assay 25

3'→5' Exonuclease Activity Assay 26

Stopped-Flow Single-Turnover Excision Reactions 28

Temperature Sensitivity of the D131G-DNA polymerase 31

RESULTS 32

Isolation of T4 DNA Polymerase Mutator Mutants 32

Construction and In Vivo Characterization of Mutant T4 Phage Strains 32

Purification of Mutant DNA Polymerases 36

DNA Polymerase Activity In Vitro 36

Exonuclease Activity of T4 DNA Polymerase Mutants 37

Pre-Steady State Kinetics of 3'→5' Exonuclease Activity 44

Excision of d2APMP from a Single-Stranded DNA Substrate 54

Effect of Increasing Strand Separation on Excision Rates 55

Competition Between Extension and Excision Reactions 61

Temperature Sensitivity of the D131G-DNA Polymerase 65

DISCUSSION 69

REFERENCES 78

LIST OF TABLES

Table	Description	Page
I.	*Escherichia coli* and bacteriophage T4 strains	15
II.	DNA sequences of single- and double-stranded DNA substrates used to measure 3'→5' exonuclease activity	27
III.	DNA sequences of 2AP-containing primer-templates	29
IV.	DNA replication fidelity of wild type and mutant T4 DNA polymerases	35
V.	DNA polymerase activity of wild type and mutant T4 DNA polymerases	38
VI.	Pre-steady state excision rates of T4 DNA polymerases on double-stranded 2AP-T primer-templates	53
VII.	Pre-steady state excision rates of T4 DNA polymerases on single-stranded 2AP-DNA substrates	56
VIII.	Pre-steady state exsision rates of the D131N-DNA polymerase on DNA substrates with increasing pre-formed strand separation	57
IX.	Pre-steady state exsision rates of wild type T4 DNA polymerase on DNA substrates with increasing pre-formed strand separation	58
X.	Pre-steady state exsision rates of the G255S-DNA polymerase on DNA substrates with increasing pre-formed strand separation	59

LIST OF FIGURES

Figure	Description	Page
1.	Conserved exonuclease and polymerase motifs in Family A, B, and C DNA polymerases	3
2.	Exonuclease domains of *E. coli* Klenow fragment and T4 DNA polymerase	9
3.	Exonuclease activity of wild type and mutant T4 DNA polymerases on a single-stranded DNA substrate	39
4.	Exonuclease activity of wild type and mutant T4 DNA polymerases on a double-stranded DNA substrate	42
5.	Fluorescence of free d2APMP and 2AP-DNA	45
6.	Excision of d2APMP from a 3'-terminal 2AP-T base pair by wild type and mutant T4 DNA polymerases	48
7.	Biphasic excision of d2APMP from a 3'-terminal 2AP-T base pair by the wild type T4 DNA polymerase	50
8.	Excision of d2APMP from a 3'-terminal 2AP-T base pair in the presence of deoxyribonucleoside triphosphates	63
9.	Decrease in polymerase and exonuclease activities caused by heat-inactivation of wild type and D131G-DNA polymerases	67
10.	Magnified view of the T4 DNA polymerase exonuclease active center	73

LIST OF ABBREVIATIONS

A	alanine	LB	Luria Broth
am	amber (nonsense codon-UAG)	$MgCl_2$	magnesium chloride
AMV	avian myeloblastosis virus	$MgSO_4$	magnesium sulfate
Asn	asparagine	mRNA	messenger ribonucleic acid
Asp	aspartate	N	asparagine
ATP	adenosine triphosphate	N_2	nitrogen gas
C	cysteine	NaCl	sodium chloride
$CaCl_2$	calcium chloride	NaOAc	sodium acetate
cpm	counts per minute	NaOH	sodium hydroxide
D	aspartate	$NaPP_i$	sodium pyrophosphate
dATP	deoxyadenosine triphosphate	NEN	New England Nuclear
dCTP	deoxycytidine triphosphate	NH_4OAc	ammonium acetate
ddATP	dideoxyadenosine triphosphate	$(NH_4)_2SO_4$	ammonium sulfate
ddCTP	dideoxycytidine triphosphate	*oc*	ochre (nonsense codon-UAA)
ddGTP	dideoxyguanosine triphosphate	OD	optical density
ddNTP	dideoxynucleoside triphosphate	OH	hydroxide
ddTTP	dideoxythymidine triphosphate	PEG	polyethylene glycol
dGTP	deoxyguanosine triphosphate	pp	pages
DNA	deoxyribonucleic acid	psi	pounds per square inch
dNTP	deoxynucleoside triphosphate	RNA	ribonucleic acid
DTT	dithiothreitol	RNAse	ribonuclease
dTTP	deoxythymidine triphosphate	rpm	revolutions per minute
E	glutamate	S	serine
EDTA	ethylenediaminetetraacetic acid	SDS	sodium dodecyl sulfate
G	glycine	Ser	serine
g43	gene *43*	su^0	supressorless
Gly	glycine	T	threonine
^3H	tritium	*Taq*	*Thermus aquaticus*
HEPES	N-[2-Hydroxyethyl]piperazine - N'-[2-ethanesulfonic acid]	Tris-HCl	Tris Hydroxymethyl Aminomethane Hydrochloride
HIV-1	Human Immunodeficiency Virus-1	*ts*	temperature sensitive
		Tyr	tyrosine
K_2HPO_4	dibasic potassium phosphate	Y	tyrosine
KH_2PO_4	monobasic potassium phosphate	$ZnSO_4$	zinc sulfate

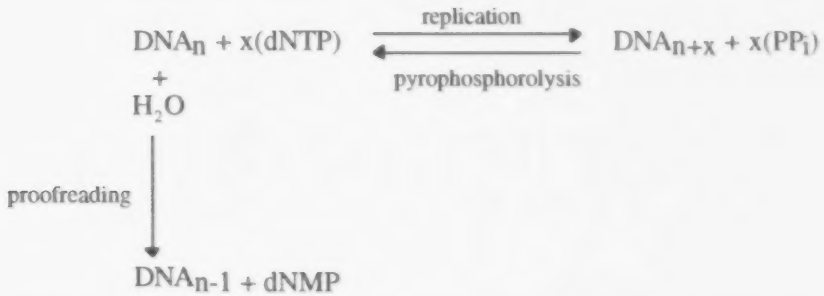
LIST OF SYMBOLS

α	alpha
β	beta
ε	epsilon
ϕ	phi
g	gravitational force
γ	gamma
λ	lambda

INTRODUCTION

The most fundamental process of life is the faithful transmission of genetic information from parent to offspring. Genetic integrity is maintained largely by DNA polymerases, the enzymes that replicate DNA by catalyzing the template-directed incorporation of deoxyribonucleotides. High fidelity DNA replication is achieved by accurate nucleotide incorporation, with an error frequency of only 10^{-5} to 10^{-6}, and the proofreading activity associated with most DNA polymerases, which increases fidelity a further 100-fold or more for an overall error frequency of 10^{-8} (Schaaper, 1993).

The reactions catalyzed by DNA polymerases can be summarized by the reaction scheme shown below, where n is the length, in nucleotides, of the primer strand.



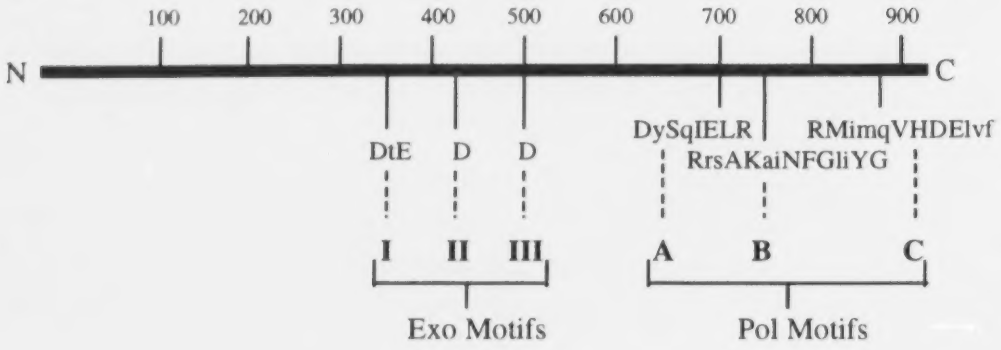
In the forward reaction, the DNA polymerase catalyzes the nucleophilic attack by the oxygen atom of the 3'-OH primer-terminus on the innermost (α) phosphorus atom of the incoming deoxyribonucleoside triphosphate (dNTP) (Romaniuk and Eckstein, 1982). Thus, a single deoxyribonucleoside monophosphate (dNMP) is added to the growing DNA chain by the formation of a phosphodiester bond and the concomitant release of pyrophosphate (PP_i). The reverse reaction, pyrophosphorolysis, is a nucleophilic attack by PP_i on the phosphorus atom of the 3'-terminal phosphodiester bond which produces dNTP and shortens the DNA strand by one nucleotide for each PP_i consumed. The degradation of PP_i by potent cellular pyrophosphatases prevents the reverse reaction and allows the DNA replication reaction to predominate.

An alternative reaction pathway, indicated by the downward arrow, is the hydrolytic removal of the 3'-terminal dNMP by the DNA polymerase-associated 3'→5' exonuclease activity. In the competition between 5'→3' polymerase and 3'→5' exonuclease activities, the state of the primer-terminus dictates which reaction pathway will be followed. For a correctly base paired primer-terminus, the forward elongation reaction predominates, while a mispaired primer-terminus slows extension so that proofreading is the kinetically favoured reaction. Because mispaired primer-termini are preferentially excised rather than extended, the exonuclease activity is a proofreading mechanism. Highly accurate DNA replication is, therefore, achieved through the cooperation of two processes, accurate nucleotide incorporation and proofreading, both of which must be considered when studying DNA polymerase function. The goal of this investigation is to use genetic and biochemical methods to further probe the mechanism by which DNA polymerases carry out exonucleolytic proofreading.

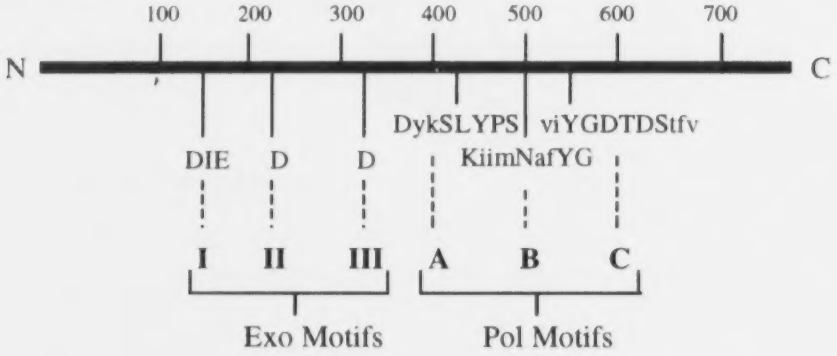
DNA polymerases are produced by all free-living organisms and also by many viruses. DNA polymerases from diverse organisms show limited amino acid sequence similarity, but several sequence motifs have been conserved throughout evolution. Three motifs in the carboxy-terminal domain of DNA polymerases (Fig. 1) are essential for 5'→3' polymerase activity (Wong *et al.*, 1988; Delarue *et al.*, 1990; Braithwaite and Ito, 1993). Highly conserved aspartate residues essential for exonucleolytic proofreading are found in three motifs (Fig. 1) in the amino-terminal domain of DNA polymerases with associated proofreading activity (Morrison *et al.*, 1991; Blanco *et al.*, 1992). Based on primary structures, DNA polymerases have been grouped by Braithwaite and Ito (1993) into three families: Family A (homology to *Escherichia coli* DNA polymerase I, encoded by the *pol*A gene); Family B (homology to *E. coli* DNA polymerase II, encoded by the *pol*B gene; and Family C (homology to *E. coli* DNA polymerase III α subunit, encoded by the *polC* [*dnaE*] gene). The higher degree of homology within a family reflects the evolutionary and functional relatedness of its members. In addition to *E. coli* DNA

Figure 1. **Conserved exonuclease and polymerase motifs in Family A, B, and C DNA polymerases.** *E. coli* DNA polymerase I, DNA polymerase II, and DNA polymerase III, α and ε subunits, are representative members of Families A, B, and C, respectively. Only the proofreading ε subunit of the DNA polymerase III holoenzyme is illustrated. Three conserved exonuclease motifs (I, II, and III) share conserved acidic active site resides (aspartate [D] and glutamate [E] residues) in all three DNA polymerase families. The consensus sequences for the three conserved polymerase motifs (A, B, and C) are given for *E. coli* DNA polymerase I and DNA polymerase II. Capital letters represent invariant residues, while non-conserved residues are given in lower case.

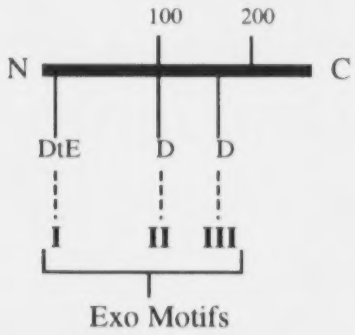
Family A - *E. coli* DNA polymerase I



Family B - *E. coli* DNA polymerase II



Family C - *E. coli* DNA polymerase III - ε subunit



polymerase I, Family A includes the DNA polymerases of bacteriophages T5 and T7, the thermostable *Taq* DNA polymerase, and the yeast mitochondrial DNA polymerase. Family B is a large group which encompasses the DNA polymerases of bacteriophages T4 and ϕ29; DNA polymerases of herpes, vaccinia, and adeno viruses; and the eukaryotic alpha, delta, and epsilon DNA polymerases. Family C is comprised of bacterial replicative DNA polymerases.

The sequence conservation among DNA polymerases implies the existence of common themes for structure and function in DNA polymerases from diverse organisms. Support for this proposal is provided by crystallographic studies which reveal structural similarities between the polymerase active centers of *E. coli* DNA polymerase I, T7 RNA polymerase, and the reverse transcriptase from HIV-1 (reviewed by Moras, 1993). Wang *et al.* (in press) have recently demonstrated considerable structural homology between the 3'→5' exonuclease domains of *E. coli* DNA polymerase I and T4 DNA polymerase, despite limited similarity at the amino acid sequence level. The evolutionary conservation of DNA polymerase structural motifs admits the possibility for structure-function characteristics of a single polymerase to be representative of DNA polymerases in general and, in particular, other members of its polymerase family. For example, the first DNA polymerase isolated, *E. coli* DNA polymerase I (Kornberg *et al.*, 1956), is a model for Family A DNA polymerases. Since its discovery, *E. coli* DNA polymerase I has been the focus of extensive biochemical characterization (reviewed by Kornberg and Baker, 1992) and genetic and structural studies (reviewed by Joyce and Steitz, 1994). The DNA polymerase from bacteriophage T4 has also been well characterized (reviewed by Kornberg and Baker, 1992) by biochemical (Capson *et al.*, 1992; Bloom *et al.*, 1994; Frey *et al.*, 1995), genetic (reviewed by Reha-Krantz, 1995), and structural studies (Wang *et al.*, in press), and thus, T4 DNA polymerase serves as a model for Family B DNA polymerases. The T4 DNA polymerase has been chosen as the model system for the present investigation into the mechanism of DNA polymerase proofreading.

Bacteriophage T4 gene *43* encodes an 898 amino acid, 103,572 dalton DNA polymerase (Spicer *et al.*, 1988). Early studies of the T4 DNA polymerase revealed that the single polypeptide contains a 5'→3' polymerase activity and a 3'→5' exonuclease activity (Goulian *et al.*, 1968; Huang and Lehman, 1972; Hershfield and Nossal, 1972). The 3'→5' exonuclease activity was subsequently demonstrated to serve a proofreading function in removing misincorporated nucleotides (Muzyczka *et al.*, 1972; Brutlag and Kornberg, 1972). The polymerization and proofreading activities of the T4 DNA polymerase are coordinated to achieve maximum replication fidelity with minimal waste of nucleotides. Extension of a correctly base paired primer-terminus is extremely rapid (400 s^{-1}) and outcompetes proofreading, which is limited by the slow rate (5 s^{-1}) at which the primer-terminus is transferred from the polymerase to the exonuclease active center, even though the hydrolysis reaction itself is rapid (100 s^{-1}) (Capson *et al.*, 1992). A 3'-terminal mispair reduces the rate of incorporation to an extent which allows the mispaired primer-terminus to undergo an intramolecular transfer from the polymerase to exonuclease active center (reviewed by Echols and Goodman, 1991). Removal of the mispaired dNMP by the 3'→5' exonuclease exposes a correctly base paired primer-terminus which is then returned to the polymerase active center (Reddy *et al.*, 1992). Thus, a delicate balance between polymerase and exonuclease activities enables T4 DNA polymerase to replicate DNA with great speed and accuracy.

The mechanism by which DNA polymerases accurately incorporate nucleotides at the 3'-terminus of a growing DNA chain has been the focus of extensive investigation (reviewed by Johnson, 1993). The incorporation of nucleotides depends on their ability to hydrogen bond with bases in the template strand. In aqueous solution, a difference in the free energy of binding between matched and mismatched base pairs at a primer-terminus is estimated in the range of 0.2 to 0.4 kcal/mol (Petruska *et al.*, 1988). If the formation of stable base pairs in solution was the basis for DNA replication, an error rate of 10^{-1} would be observed (Echols and Goodman, 1991). A much lower misincorporation frequency in

the range of 10^{-5} to 10^{-6} is measured for DNA polymerase-catalyzed DNA replication because DNA polymerases selectively incorporate correct nucleotides (Loeb and Kunkel, 1982). The DNA polymerase is able to discriminate against mismatches based on the physical properties of the terminal base pair. Watson-Crick base pairs conform to a specific geometry that is violated by non-Watson-Crick base pairs. Secondly, mismatched base pairs in aqueous solution are afforded stability by hydrogen bonding with water molecules (Johnson, 1993). DNA polymerases are proposed to amplify the free energy difference between matched and mismatched base pairs by adopting a protein conformation that restricts non-Watson-Crick base pair geometries and by excluding water from the polymerase active center (Petruska *et al.*, 1988). Consistent with this model is the observation that the DNA polymerase undergoes a rate-limiting conformational change prior to catalysis that presumably results in a tighter association between the enzyme and the incoming dNTP (Frey *et al.*, 1995). Thus, discrimination against incorrectly base paired nucleotides by the polymerase active center of DNA polymerases is a major determinant of high fidelity DNA replication.

Further accuracy in DNA replication is achieved by the exonucleolytic proofreading reaction. Structural information has recently been obtained for the exonuclease domain of the T4 DNA polymerase (Wang *et al.*,1996) and the DNA polymerase from the related bacteriophage, RB69 (J. Wang, personal communication). Prior to these studies, the Klenow fragment of *E. coli* DNA polymerase I, which lacks the 5'→3' exonuclease domain but retains the 5'→3' polymerase and 3'→5' exonuclease domains, was the only proofreading DNA polymerase for which a high resolution crystal structure had been solved (Ollis *et al.*, 1985). The crystallographic data, in conjunction with genetic and biochemical studies, have been instrumental in providing detailed structural and functional information for the Klenow fragment 3'→5' exonuclease domain (reviewed by Joyce and Steitz, 1994). Resolution of the Klenow fragment complexed with duplex DNA reveals that DNA bound in the exonuclease active site is single-stranded (Freemont *et al.*, 1988)

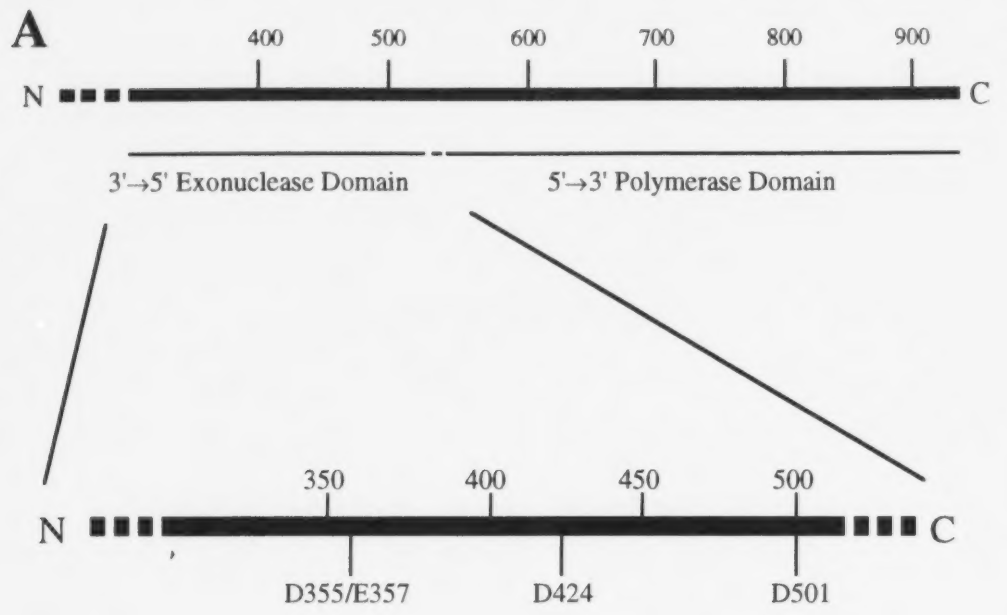
which is consistent with the earlier observation that the 3'→5' exonuclease activity of proofreading DNA polymerases is a single-stranded DNA exonuclease activity (Huang and Lehman, 1972).

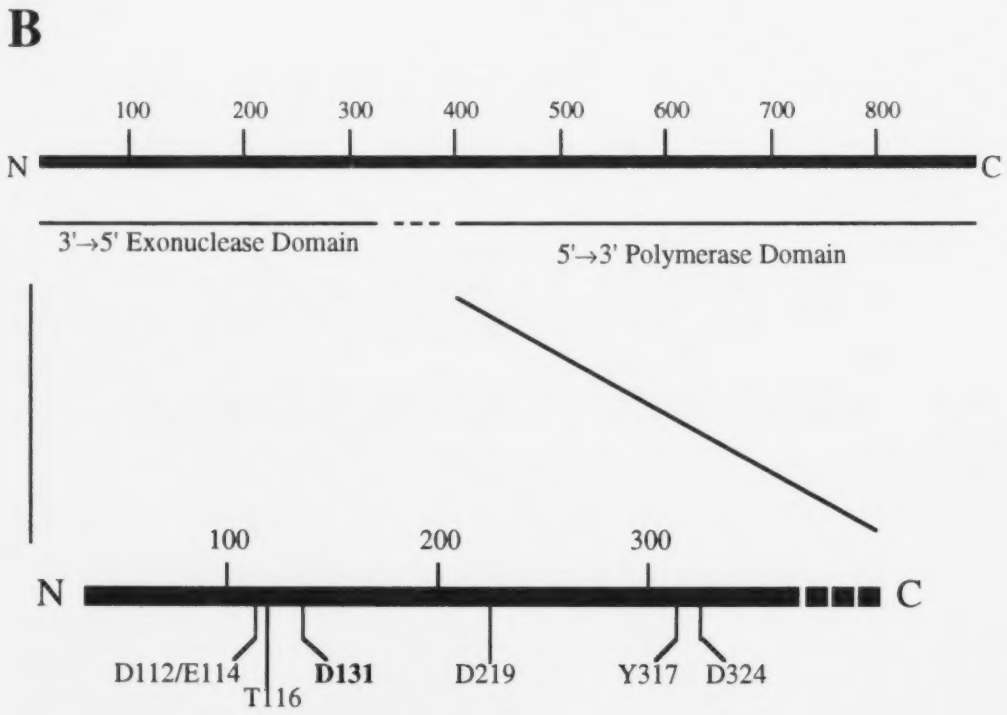
The hydrolysis of a single-stranded primer-terminus by the Klenow fragment is described in terms of a two metal ion model (Freemont *et al.*, 1988). The acidic side chains of amino acid residues in the active center of the Klenow fragment exonuclease domain coordinately bind two metal ions which are required for catalysis. A divalent metal ion is coordinated at metal ion site A by residues D355, E357 and D501 (Fig. 2A), together with an oxygen atom from the 5' (scissile) phosphate of the 3'-terminal dNMP (Derbyshire *et al.*, 1988). Metal ion A contributes to catalysis by binding a hydroxyl ion in an orientation that promotes its nucleophilic attack on the phosphorus atom of the scissile phosphate (Beese and Steitz, 1991). A second divalent metal ion, coordinated at metal ion site B by residues D424, D355 (Fig. 2A) and two oxygen atoms from the scissile phosphate, is proposed to stabilize the transient pentacovalent intermediate and the leaving 3' oxyanion (Beese and Steitz, 1991).

A general mechanism for exonucleolytic proofreading is implied by the conservation of acidic active site residues in other polymerases that possess a 3'→5' exonuclease function (Morrison *et al.*, 1991; Blanco *et al.*, 1992). Exonuclease active site residues, D112, E114, D219, and D324 (Fig. 2B) in T4 DNA polymerase are analogous to *E. coli* DNA polymerase I residues D355, E357, D424, and D501, respectively. Mutant T4 DNA polymerases in which an alanine residue has been substituted for one or more of the acidic active site residues are deficient in 3'→5' exonuclease activity, but retain 5'→3' polymerase activity (Reha-Krantz *et al.*, 1991; Reha-Krantz and Nonay, 1993; Frey *et al.*, 1993).

DNA polymerases with reduced exonuclease activity due to the D112A+E114A, D219A, or D324A amino acid substitutions increase spontaneous mutation frequencies by a factor of 60- to 500-fold *in vivo* (Reha-Krantz and Nonay, 1993). This increase in the

Figure 2. **Exonuclease domains of *E. coli* Klenow fragment and T4 DNA polymerase.** The Klenow fragment (**A**) lacks the 5'→3' exonuclease domain of *E. coli* DNA polymerase I, but retains both the 3'→5' exonuclease and the 5'→3' polymerase domains (residues 324 to 928). The positions of the highly conserved acidic active site residues, D355, E357, D424, and D501, of the Klenow fragment 3'→5' exonuclease domain are indicated. The analogous acitve site residues, D112, E114, D219, and D324, of the T4 DNA polymerase are also shown (**B**). The positions of D131 and the residues with which it interacts, T116 and Y317, are illustrated in relation to the exonuclease active site residues of the T4 DNA polymerase.


A
400
500
600
700
800
900
N
C
3'→5' Exonuclease Domain
5'→3' Polymerase Domain
350
400
450
500
N
C
D355/E357
D424
D501


B
100
200
300
400
500
600
700
800
N
C
3'→5' Exonuclease Domain
5'→3' Polymerase Domain
100
200
300
N
C
D112/E114
T116
D131
D219
Y317
D324

frequency of DNA replication errors is referred to as a mutator phenotype. Amino acid substitutions that give rise to a mutator phenotype are not confined to exonuclease active site residues, but include residues throughout the DNA polymerase. Biochemical studies of classical conditional lethal mutants of T4 have identified mutators with increased misinsertion frequency, such as *ts*L88 (Hershfield, 1973) due to an amino acid substitution in the polymerase domain (Reha-Krantz, 1989), and mutators with decreased exonuclease activity, such as *ts*L56 and *ts*L98 (Hall and Lehman, 1968; Muzyczka *et al.*, 1972) caused by amino acid substitutions in the exonuclease domain (Reha-Krantz, 1989). Thus, mutations that affect either the 5'→3' polymerase or 3'→5' exonuclease reactions can confer a mutator phenotype, since both nucleotide incorporation and exonucleolytic proofreading contribute to DNA replication fidelity.

The importance of non-active site residues in maintaining the high fidelity of DNA replication reflects the involvement of these residues in other, non-catalytic steps of the proofreading mechanism. In addition to mutations that affect the chemistry of the polymerase and exonuclease reactions, a mutator phenotype can result from mutations that decrease the frequency at which the primer-terminus is transferred from the polymerase to exonuclease active center. These active site "switching" mutants can arise from amino acid substitutions that increase the stabilization of both correct and mispaired primer-termini in the polymerase active center or decrease the efficiency with which a mispaired primer-terminus is strand separated and positioned within the exonuclease active center (Stocki *et al.*, 1995; Marquez and Reha-Krantz, 1996).

Over 20 T4 DNA polymerase mutator mutants were isolated in genetic selections for mutants with high spontaneous mutation frequencies (Reha-Krantz *et al.*, 1986; Reha-Krantz, 1988). Among these mutants is the *tsmel5* strain, renamed D131G to indicate the aspartate to glycine amino acid substitution encoded by the base substitution mutation in gene *43* (Reha-Krantz, 1988). Based on the location of D131 in the exonuclease domain of the T4 DNA polymerase, the mutator activity of the D131G-DNA polymerase mutant is

predicted to result from a decrease in 3'→5' exonuclease activity. Although D131 is not one of the highly conserved active site residues, the mutator activity of the D131G-DNA polymerase mutant is comparable to that of mutants with alanine substituted for active site aspartate residues. The involvement of carboxylate residues in coordinating divalent metal ions required for catalysis, and the high mutator activity caused by the D131G substitution, suggested the possibility that D131 could represent a previously unidentified ligand for a divalent metal ion. An alternative hypothesis, supported by the temperature sensitivity of the D131G-DNA polymerase mutant *in vivo*, was that D131 is a residue important for maintaining the three-dimensional conformation of the exonuclease active center, since replacement of an acidic aspartate residue with a small, neutral glycine residue is expected to disrupt protein structure. The purpose of this study is to uncover the importance of D131 for exonuclease activity as a means of extending our knowledge of the exonucleolytic proofreading mechanism.

The functional significance of D131 was reinforced by the isolation of another mutator mutant, the D131N-DNA polymerase mutant, in which the aspartate residue was replaced by an asparagine residue (Reha-Krantz, 1988). The D131N-mutant strain has a weaker mutator phenotype than the D131G-strain and is not temperature sensitive. A third mutant, the D131S-DNA polymerase mutant, with serine substituted for the aspartate residue, was identified as a spontaneous revertant of the temperature sensitive phenotype of the original D131G-mutant strain (Reha-Krantz, unpublished observation). The proposed involvement of D131 in proofreading was, therefore, investigated by comparing the effects of D131G, D131N and D131S amino acid substitutions on T4 DNA polymerase 3'→5' exonuclease activity.

The role D131 plays in maintaining high fidelity during replication by T4 DNA polymerase is difficult to determine based solely on *in vivo* phenotypes of the DNA polymerase mutants because a wide range of functional defects can give rise to a mutator phenotype. While not an exact recreation of *in vivo* conditions, biochemical assays

performed on purified enzymes *in vitro* are an efficient way to determine alterations in function of the mutant DNA polymerases. The D131G, D131N and D131S substitution mutations were introduced into the T4 DNA polymerase expression vector (Lin *et al.*, 1987) by site-directed mutagenesis. The mutant proteins were overexpressed and purified to near homogeneity. The enzymatic activities of the D131G-, D131N- and D131S-mutant DNA polymerases were compared to the exonuclease deficient D112A+E114A mutant polymerase and to the wild type enzyme. Biochemical characterization of the mutant enzymes employed several enzyme assays with radioactively labeled substrates as well as a more sensitive fluorescence assay that measures the exonuclease reaction on a millisecond time scale. The data support a role for D131 in maintaining the structural integrity of the exonuclease active center. This finding is consistent with recent crystallographic studies of the T4 DNA polymerase exonuclease domain which reveal a potential hydrogen bond between residues D131 and Y317 and residues D131 and T116 (J. Wang and T. Steitz, personal communication). The contribution of D131 to the protein conformation of the exonuclease active center, reflects the power of genetic selection in identifying amino acid residues that are essential to the proper function of the 3'→5' exonuclease activity of T4 DNA polymerase.

MATERIALS AND METHODS

Microbiological Media

Hershey broth, bottom-layer agar, and top-layer agar were prepared according to Chase and Doermann (1958) with the addition of 1 g sodium citrate/liter to the bottom agar. M9$^+$ medium, consisting of M9 (Adams, 1959) supplemented with 0.4% glucose, 0.5% Difco casamino acids, and 1.1 mM $MgSO_4$ was used in the preparation of phage stocks.

Growth of Bacteria and Bacteriophage

The *Escherichia coli* and bacteriophage T4 strains used are described in Table I. The *E. coli* strain CR63 was used as the host for preparation of T4 phage cultures as previously described (Reha-Krantz and Lambert, 1985). High titer cultures (approximately 1×10^{11} phage/mL) were obtained by inoculating 10 mL cultures of CR63 at 2×10^8 cells/mL M9$^+$ with individual 12- to 18-h plaques, followed by incubation with aeration (by shaking) overnight at 30°C. T4 gene *43-rII* double mutants were constructed by recombination and selected by the method of Doermann and Boehner (1970). An exponential culture of CR63 (2×10^8 cells/mL in M9$^+$) was infected at a multiplicity of 5 for each parental phage and aerated by gentle shaking at 30°C. At 12 min post-infection, the culture was diluted 1000-fold to reduce the concentration of unabsorbed phage in the medium. After an additional 90-min incubation, chloroform was added to complete lysis and to sterilize the culture. Phage progeny were titered on the CR63 permissive host to determine the total number of progeny. Titers on hosts restrictive for one or both parents indicated the relative numbers of parental-type progeny and wild type recombinants, respectively. Temperature sensitive mutants of gene *43* were restricted by plating at 42°C, gene *43* amber mutants were restricted by plating on a non-suppressor host (*E. coli* B), and *rII*$^-$ mutants were restricted by plating on a host carrying a lambda lysogen (CR63[λh]). Doubly mutant recombinants were detected by replica plating onto the permissive and restrictive hosts (only CR63 supports growth of doubly mutant phage).

Table I. *Escherichia coli* and bacteriophage T4 strains

Strain	Genotype	Relevant Characteristics and Reference/Source
E. coli		
CR63	K strain, *supD*	Permissive for all T4 strains, amber suppressor, inserts serine (Appleyard *et al.*, 1956)
CR63 (λh)	λ lysogen of CR63	Restricts T4 *rII* mutant phage
B	B strain	Restricts T4 *am* mutant phage
CJ236	*dut1 ung1 thi1 relA1* [F':*pCJ105 (Cm^r)*]	lacks dUTPase and uracil N-glycosylase activities, permissive for M13 (Joyce and Grindley, 1984)
DH5α	*endA1 hsdR17* ($r_K^- m_K^+$) *supE44 thi1 recA1 gyrA* (Nal^R) *relA1* Δ*(lacZYA-argF) U169 deoR* (ϕ80 Δ*(lacZ)* M15)	active dUTPase and uracil N-glycosylase (degrades uracil-containing DNA) (Woodcock *et al.*, 1989)
BL21(DE3)	F$^-$ *ompT hsd* S_B *gal* (*cI ts857 ind1 sam7 nin5 lacUV T7 gene 1*)	T7 RNA polymerase gene under control of the inducible *lacUV5* promoter (Studier and Moffat, 1986)
NapIV	B^E *hsd*$_M$K$^+$ *hsd*$_R$K$^-$ *hsd*$_S$K$^+$ mal$^+$ - $\lambda^S r_{G1}$ (B_1^-)	host strain used in isolation of total nucleic acid from T4 for RNA sequencing (Nelson *et al.*, 1982)
Bacteriophage T4		
T4B *rIIUV199oc*	*rIIB ochre* mutation	restricted on λ lysogens (Drake, 1963)
*ts*A60	*g43 ts* DNA pol mutant	restricted at 42°C (Epstein *et al.*, 1964)
Y128*am-rIIUV199oc*	*g43* 382TAT→382TAG 128Tyr→128am, *rIIB ochre* mutation	Amber mutation near codon 131 (constructed by site-directed mutagenesis of cloned gene *43* and introduced to T4 by marker rescue)
D131G-*rIIUV199oc*	*g43* 391GAT→391GGC 131Asp→131Gly, *rIIB ochre* mutation	Strong mutator; temperature sensitive (constructed by marker rescue of Y128*am-rUV199oc*)
D131N-*rIIUV199oc*	*g43* 391GAT→391AAT 131Asp→131Asn, *rIIB ochre* mutation	Weak mutator (constructed as above)
D131S-*rIIUV199oc*	*g43* 391GAT→391AGC 131Asp→131Ser, *rIIB ochre* mutation	Weak mutator (constructed as above)

Construction of T4 DNA Polymerase Mutants

Mutations specifying the D131G, D131N, and D131S substitutions, and the nonsense mutation, Y128*am*, were introduced into the cloned T4 DNA polymerase gene carried on the pTL7-g*43* vector (Lin *et al.*, 1987). The pTL7-g*43* vector is a pBR322 derivative with both ColE1 and F1 origins of replication, an ampicillin-resistance selectable marker, and the T4 gene *43* under control of the T7 promoter. Site-directed mutagenesis of gene *43* was performed using a modification of the Kunkel method which employs a strong biological selection against uracil-containing template DNA to enrich for the mutation-containing complimentary strand (Kunkel *et al.*, 1987). Uracil-containing template for site-directed mutagenesis was produced by propagating the pTL7-g*43* vector in *E. coli* strain CJ236 which lacks dUTPase and uracil *N*-glycosylase activities. A 250 mL culture of CJ236 was grown at 37°C to early log phase in LB medium containing 30 μg/mL chloramphenicol (selection for the F' factor) and 50 μg/mL ampicillin (selection for the pTL7-g*43* vector). M13K07 helper phage were added to a multiplicity of infection of 20. At 1 h post-infection, a final concentration of 70 μg/mL kanamycin was used to select for cells containing helper phage.

Infected cells were incubated overnight at 30°C. The cells were then pelleted by centrifugation in a Sorvall GSA rotor at 10,000 rpm (16,000 × *g*) at 4°C for 15 min. The supernatant, containing the uracil-substituted phagemids, was treated with RNAse at a final concentration of 5 μg/mL, and then recentrifuged as described above. Solid NaCl was added to give a final concentration of 0.5 M and then the mixture was incubated for 1 h at 0°C. Solid PEG-8000 (BDH) was added (final concentration of 3%) over a period of 1 h at 0°C to precipitate the phagemids. Phagemids were collected by centrifugation as described above and the pellet was resuspended in 6 mL cold 50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 100 mM NaCl. Two phenol:chloroform (50% phenol, 48% chloroform, 2% isoamyl alcohol; equilibriated with 0.1 M Tris-HCl [pH 8.0]) extractions and two extractions with chloroform:isoamyl alcohol (24:1) were performed. The phagemid DNA

was precipitated by adding 2 volumes of 95% ethanol and allowing precipitation to continue overnight at -20°C. The DNA was collected by centrifugation in a Sorvall SS-34 rotor at 10,000 rpm (12,000 × *g*) for 15 min at 4°C, washed with 70% ethanol, recentrifuged and then resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Oligonucleotides containing the desired base substitutions and a 5' phosphate residue were purchased from the DNA Synthesis Lab, Department of Biological Sciences, University of Alberta. An annealing mixture, containing 6 pmol of oligonucleotide, 3 pmol of uracil-containing template DNA, and 100 mM NaCl in 30 µL total volume, was incubated in a 1.5 mL Eppendorf tube in a 1 L water bath at 70°C for 5 min and then allowed to cool slowly to room temperature (about 3 h). Brief centrifugation (13,000 × *g* for 30 sec) was used to collect condensation before placing the annealed primer-template on ice. Synthesis of the complementary strand and subsequent ligation to form a covalently closed circular DNA molecule was accomplished by T4 DNA polymerase and T4 DNA ligase, respectively. A 20 µL synthesis reaction mixture contained 2 µL of annealed primer-template (0.2 pmol), standard T4 DNA polymerase buffer (67 mM Tris-HCl [pH 8.8], 16.7 mM $(NH_4)_2SO_4$, 0.5 mM DTT), a 200 µM dNTP mix, 200 µg bovine serum albumin, 4 pmol T4 DNA polymerase, and 6.67 mM $MgCl_2$. The mixture was incubated 5 min at room temperature in the absence of $MgCl_2$ to allow stable complex formation. DNA replication was initiated by addition of $MgCl_2$. Synthesis proceeded at room temperature for 5 min before the addition of 2 units of T4 DNA ligase (7 units/µL, Pharmacia) and 1 µL of 5 mM ATP and a further 10 min incubation at 42°C. The reaction was terminated by the addition of EDTA to a final concentration of 16.7 mM.

Reaction products were examined by electrophoresis of 9 µL of the reaction mix with 1 µL of 10X loading buffer (0.42% bromophenol blue, 50% glycerol) in a 0.5% agarose gel in 1X TBE buffer (100 mM Tris base, 100 mM boric acid, 2.5 mM EDTA), containing 0.5 µg/mL ethidium bromide. Single-stranded and double-stranded phagemid

DNA controls were run as size standards. Little or no primer extension was detected in control reactions in which either the oligonucleotide primer or T4 DNA polymerase had been omitted. A control reaction with no DNA ligase produced a double-stranded molecule which migrated like nicked double-stranded phagemid DNA. Ligated reaction products migrated with a mobility identical to that of the covalently closed double-stranded phagemid DNA.

Site-directed mutagenesis reaction products were transformed into a *dut*$^+$*ung*$^+$ strain of *E. coli* (DH5α) which had been made competent by the $CaCl_2$ method (Sambrook *et al.*, 1989; pp 1.82-1.84). Log phase cells were pelleted by centrifugation in a Sorvall SS-34 rotor at 5,000 rpm (3,000 × *g*) for 5 min at 4°C and resuspended in 25 mL of ice cold 50 mM $CaCl_2$. After a 30 min incubation at 0°C, the cells were centrifuged as above and the pellets were resuspended in 5 mL of cold 50 mM $CaCl_2$. Competent cells were stored at 4°C and used within 24 h of the initial treatment with $CaCl_2$. Approximately 40 ng of double-stranded DNA (contained in 2 μL of the mutagenesis reaction mixture) was added to 0.3 mL of competent DH5α cells. A control transformation with purified double-stranded phagemid DNA was carried out in parallel to assess transformation efficiency. The mixture of cells and DNA was incubated at 0°C for 40 min, heat shocked at 42°C for 2 min, then returned to 0°C for 5 min. After the addition of 0.7 mL LB, the cells were allowed to express the β-lactamase gene during a 1 h incubation at 37°C. Transformants were selected by plating 100 μL of the transformation mixture on LB plates containing a final concentration of 50 μg/mL ampicillin. From a typical plate containing 100 colonies, 5 to 10 transformants were screened for the desired mutation.

Confirmation of T4 DNA Polymerase Mutations

The base substitution mutations introduced by site-directed mutagenesis created an amino acid change in the protein coding sequence of the T4 DNA polymerase gene, as well as a new restriction endonuclease cleavage site. Ampicillin-resistant transformants were

screened by restriction endonuclease mapping. The mutagenized phagemid vectors were isolated from DH5α cells by the alkaline lysis method for plasmid DNA extraction (Sambrook *et al.*, 1989; pp 1.25-1.28). The cells from 1.5 mL of a stationary phase culture were pelleted by centrifugation (13,000 × *g* for 5 min) and resuspended in 100 µL of lysis buffer (25 mM Tris-HCl [pH 8.0], 50 mM glucose, 10 mM EDTA, 4 mg/mL egg white lysozyme [Worthington]). The mixture was incubated at room temperature for 5 min before adding a 200 µL solution of 0.2 M NaOH and 1% SDS and incubating for an additional 5 min at 0°C. A 150 µL solution of ice cold 5 M potassium acetate was added to precipitate cell debris. After a 5-min incubation at 0°C, the precipitate was pelleted by centrifugation as described above. The supernatant was extracted twice with an equal volume of phenol:chloroform (50% phenol, 48% chloroform, 2% isoamyl alcohol; equilibriated with 0.1 M Tris-HCl [pH 8.0]). Phagemid DNA was precipitated with two volumes of 95% ethanol, washed with 70% ethanol and the dried pellets were resuspended in 50 µL TE and 20 µg/mL RNAse A.

Phagemid DNA was digested with 1 unit of *Hae*II or *Ase*I, restriction endonuclease markers for mutations conferring the D131G and D131S, and the D131N amino acid substitutions, respectively. Reactions were allowed to proceed for 1 h at 37°C prior to addition of loading buffer (0.42% bromophenol blue, 50% glycerol). Reaction products were separated by electrophoresis in a 1% agarose gel containing 0.5 µg/mL ethidium bromide. Mutants were easily identified by comparing the restriction pattern of mutagenized phagemids to that of the wild type pTL7-g*43* vector.

The sequence of RNA transcripts produced by the mutated T4 DNA polymerase gene was determined to confirm the presence of desired mutations and the absence of any additional mutations that may have arisen during the site-directed mutagenesis procedure. Phagemid DNA isolated from DH5α cells was transformed (as described above) into the *E. coli* expression host, BL21(DE3) (Studier and Moffat, 1986). This strain is a λ lysogen which contains the T7 RNA polymerase gene under control of the inducible *lacUV5*

promoter. High-level expression of T4 DNA polymerase from a T7 promoter on the pTL7-g*43* construct is achieved by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) which induces the production of T7 RNA polymerase from the *lac*UV5 promoter.

T4 DNA polymerase mRNA for sequencing was isolated by a modification of the phage mRNA extraction procedure previously described (Reha-Krantz, 1987). A culture of early log phase BL21(DE3) cells transformed with a mutant T4 DNA polymerase phagemid construct was induced by the addition of 0.3 mM IPTG. Induction enriches for gene *43* mRNA transcripts among the total nucleic acid isolated from the cells. At 30 min post-induction, cells were chilled on ice then pelleted by centrifugation in a Sorvall SS-34 rotor at 10,000 rpm (12,000 × *g*) for 15 min at 4°C. The pellet was resuspended in 225 µL of a cold 0.9% saline solution and transferred to an Eppendorf tube. The cells were lysed with 25 µL lysis buffer (0.5 M Tris-HCl [pH 6.8], 20 mM EDTA, 10% w/v SDS) for 3 min at 68°C. A 1 M solution of NaOAc [pH 5.2] was added to give a final concentration of 0.2 M. The lysate was extracted three times with an equal volume of phenol:chloroform (50% phenol, 48% chloroform, 2% isoamyl alcohol; equilibriated with 0.2 M NaOAc [pH 5.2]) and once with an equal volume of chloroform:isoamyl alcohol (24:1). A one-tenth volume of 5 M NH_4OAc and 2.5 volumes of cold 95% ethanol were added, followed by an overnight precipitation at -20°C. The precipitate was collected by centrifugation (13,000 × *g* for 15 min). The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 30 µL of 1 mM EDTA.

The T4 DNA polymerase gene was sequenced using 14 synthetic oligonucleotide primers that span the length of the mRNA transcript. Primers were 5'-end-labeled in a reaction containing 10 pmol primer, 10 pmol [γ-^{32}P]ATP (NEN, 1000-3000 Ci/mmol), 2 units T4 polynucleotide kinase (Pharmacia, 9.7 units/µL), and 1 µL 10X kinase buffer (0.5 M Tris-HCl [pH 7.6], 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA). The reaction was incubated at 37°C for 30 min. Labeled primers were stored at -20°C and could be used for a two-week period.

The annealing reaction, containing 9 µL of total nucleic acid extracted from induced cells, 0.7 µL (approximately 0.7 pmol) end-labeled primer, and 2.4 µL 5X annealing buffer (250 mM Tris-HCl [pH 8.3], 300 mM NaCl, 50 mM DTT), was incubated at 60°C for 3 min, frozen on dry ice for 1 min, and thawed on ice. Sequencing reactions contained 2 µL of the above primer-template mix, 1 µL dNTP mix (all 4 dNTPs at 2 mM in annealing buffer), 1 µL ddNTP (either 250 µM ddATP, ddCTP, ddGTP, or ddTTP), and 1 µL AMV reverse transcriptase mix [3.4 µL AMV reverse transcriptase (Boehringer Mannheim, 25 units/ µL), 8.5 µL 5X reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 300 mM NaCl, 50 mM DTT, 150 mM $MgCl_2$), 30.6 µL water]. The reactions were incubated for 30 min at 48°C and were terminated by adding 5 µL loading dye (94 µL deionized formamide, 4 µL 10X Tris-borate-EDTA gel buffer, 2 µL 2% bromophenol blue/xylene cyanol blue). Samples were heated at 95°C for 5 min prior to loading on a standard 7% polyacrylamide, 8 M urea sequencing gel. Dried gels were exposed to x-ray film or a phosphor screen.

Purification of T4 DNA Polymerase Mutant Enzymes

Mutant enzymes containing the D131G, D131N, or D131S amino acid substitutions, were overproduced and purified from BL21(DE3) cells containing the modified T4 DNA polymerase expression vectors. Culture medium (50 liters) contained 400 g of tryptone, 160 g of yeast extract, 400 g of K_2HPO_4, and 40 g of KH_2PO_4. Sterilized medium was supplemented with 2 liters of sterile 20% glucose to a final concentration of about 0.8%. Ampicillin, $ZnSO_4$, and $MgSO_4$ were added to give final concentrations of 50 µg/ml, 0.08 mM and 0.8 mM, respectively. A four-liter inoculum of early log phase cells was added to a fermentor containing medium prewarmed to 35°C. At an OD_{590} of 0.3, the temperature was shifted to 30°C and when an OD_{590} of 0.4 was reached, 0.2 mM IPTG was added. At 3 h post-induction, the culture was concentrated by Millipore filtration and cells were pelleted by Sharples centrifugation.

T4 DNA polymerase was purified according to published procedures (Reha-Krantz *et al.*, 1993). Frozen cells (60 to 70 g) from the induced fermentor culture were partially thawed in a Waring blender jar at 4°C and then suspended in 300 mL of cold TES buffer (40 mM Tris-HCl [pH 8.1], 2 mM EDTA, 25% sucrose) and 14 mM β-mercaptoethanol. The cells were stirred briefly and then homogenized by several short pulses in the blender. Lysis was furthered by the addition of egg white lysozyme to give a final concentration of 0.5 mg/mL (225 mg of lysozyme [Worthington] dissolved in 150 mL of TES buffer). The resulting viscous solution was stirred occasionally for 30 min at 4°C before adding a 450 mL solution of 0.1% Brij 58 (polyoxyethylene 20 cetyl ether [Sigma]), 50 mM Tris-HCl [pH 8.1], and 7 mM β-mercaptoethanol. Stirring of the viscous cell lysate was continued for an additional 30 min at 4°C, prior to centrifugation in a Beckman 50.2 Ti rotor at 40,000 rpm (150,000 × *g*) for 30 min at 6°C .

The clear supernatant was partially purified of nucleic acids by streptomycin sulfate precipitation. Dropwise addition of a 20% streptomycin sulfate solution to a final concentration of 4% gave a milky white suspension which was stirred gently for an additional 30 min at 4°C. Centrifugation in a Sorvall GSA rotor at 8,500 rpm (12,000 × *g*) for 30 min at 6°C removed the precipitate.

The gradual addition of 40 g ammonium sulfate/100 mL of supernatant was followed by at least 1 h of additional stirring. The $(NH_4)_2SO_4$ precipitate was collected by centrifugation in the Beckman 50.2 Ti rotor at 45,000 rpm (180,000 × *g*) for 30 min at 6°C. The pellet was resuspended in one tenth of the pre-centrifugation volume of A_0 buffer (40 mM Tris-HCl [pH 7.4], 50 mM NaCl, 10% glycerol, 0.1 mM EDTA, 10 mM β-mercaptoethanol) and dialyzed (dialysis bags, Spectra/Por Membrane; molecular weight cutoff, 20,000) against two changes of 4-liter volumes of A_0 buffer. A small amount of precipitate formed during dialysis and was removed by centrifugation in the Beckman 50.2 Ti rotor as described above.

The clear $(NH_4)_2SO_4$ fraction was loaded by gravity onto a Q-Sepharose (Pharmacia) column (75 mL; 5-cm diameter). A Pharmacia fast-performance liquid chromatography apparatus was used to run the column at 5 mL/min. A stepwise increase in the salt concentration (0.050 M; 0.080 M; 0.150 M; 0.200 M; 0.500 M) of the A_0 buffer was used to elute discrete fractions, with the T4 DNA polymerase activity eluting at 0.15 M NaCl.

The Q-Sepharose fraction was further purified by a phosphocellulose (P-11; Whatman) column (40 mL; 5-cm diameter). The column was run at 5 mL/min, and 5 mL fractions were eluted by the stepwise increase in the salt concentration (0.050 M; 0.190 M; 0.225 M; 0.275 M; 0.300 M; 0.350 M; 0.500 M) of the A_0 buffer. The peak of T4 DNA polymerase activity was recovered in fractions eluted at 0.275 M NaCl. The purified enzyme, after being dialyzed against buffer containing 50% glycerol, 2 mM DTT, 20 mM potassium phosphate buffer [pH 6.5], and 0.1 mM EDTA, was stored at -20°C.

Polymerase preparations were homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining. The single, visible band had a mobility consistent with the molecular weight of T4 DNA polymerase (103,572 daltons). The concentration of T4 DNA polymerase was measured spectrophotometrically using the experimentally determined molar extinction coefficient at 280 nm (εM_{280}) of 1.49 x 10^5 M^{-1} cm^{-1} (Reha-Krantz *et al.*, 1991).

Transfer of Constructed Mutations into Bacteriophage T4 by Homologous Recombination

Homologous recombination was used to introduce T4 DNA polymerase mutations (constructed by site-directed mutagenesis) directly into the phage genome. During T4 infection of *E. coli* cells carrying the pTL7-g*43* phagemid construct, recombination occurs between the phage gene *43* and the cloned copy of the gene on the phagemid. If the infecting phage has a conditional lethal mutation in gene *43*, the mutant phenotype will be "rescued" by the reciprocal exchange of phagemid DNA for phage DNA carrying the

mutation. Approximately 1% of the progeny will be recombinant. In this way, a mutation present on the phagemid can be transferred to the phage genome. Marker rescue is optimized by selecting a phage mutation close enough to the phagemid mutation that the segment of DNA exchanged has a high probability of always spanning both mutations.

The Y128*am* mutation was introduced into T4 phage by marker rescue with the T4 *ts*A60 mutant (codon 172). An exponentially dividing culture of DH5α, containing the Y128*am* mutant phagemid construct, was infected with *ts*A60 at a multiplicity of infection of 5. Infected cells were aerated by gentle shaking at 30°C for 12 min and then diluted 1000-fold. After incubating an additional 90 min, the culture was lysed with chloroform. Total progeny were titered on CR63 at 30°C and recombinants were selected on CR63 at 42°C, the nonpermissive temperature for *ts*A60. Recombinants were screened for the presence of the Y128*am* mutation (by replica plating on the permissive CR63 [*supD*] host and restrictive B [su^0] host).

The Y128*am* phage strain provided a reliable marker for the rescue of the three mutations at codon 131 (D131G, D131N, and D131S). An additional mutation at the *rIIB* locus was desired in these phage for use in mutation frequency analysis. Thus, prior to marker rescue, Y128*am* was constructed with the *rIIUV199oc* mutation as described above. The subsequent rescue of codon 131 mutations produced doubly mutant phage (g*43* D131G-*rIIUV199oc*, g*43* D131N-*rIIUV199oc*, and g*43* D131S-*rIIUV199oc*). The T4 gene *43* mRNA transcripts from mutant phage were sequenced to ensure that only the desired mutations were present. Total nucleic acid was purified from T4 mutant phage-infected NapIV cells. An exponential culture of NapIV (3×10^8 cells/mL M9+) was infected with T4 phage at a multiplicity of infection of 10. At 10 min post-infection, 10 mL of infected cells were chilled on ice and then pelleted by centrifugation in a Sorvall SS-34 rotor at 10,000 rpm (12,000 × g) for 15 min at 4°C. Nucleic acid was purified and sequenced as described above.

Characterization of T4 DNA Polymerase Mutants

Spontaneous mutation frequencies were determined by measuring the number of revertants at the *rIIUV199oc* site (Reha-Krantz and Nonay, 1993) and in a forward mutation assay that detects mutants resistant to acriflavine (Reha-Krantz and Lambert, 1985). Mutator DNA polymerases increase the number of *rII*$^+$ revertants and acriflavine-resistant mutants compared to wild type DNA polymerases. The number of *rII*$^+$ revertants was measured by titering mutant cultures on a λ lysogenic strain, CR63 (λh), which restricts the growth of T4 *rII*$^-$ mutant phage, but not *rII*$^+$ revertant phage. Mutation frequency was then determined by comparing the number of *rII*$^+$ revertants to the total number of phage (measured by plating on the permissive host, CR63). A second measure of mutation frequency was provided by comparison of the number of acriflavine-resistant mutants on plates containing 0.5 μg acriflavine/mL to the total number of phage on non-acriflavine-containing plates. For each assay, an index of mutator activity was derived from the median mutation frequency of five individual cultures (Luria and Delbrück, 1943).

Mutant T4 phage were assayed for their sensitivity to high temperatures. Whereas most T4 phage strains are viable at 42°C, growth of temperature sensitive mutant phage is restricted. Mutants were considered temperature sensitive if titers were significantly lower (by at least four orders of magnitude) on the permissive host, CR63, at 42°C compared to 30°C.

T4 DNA Polymerase 5'→3' Polymerase Activity Assay

DNA polymerase activity was measured using a template of "activated" DNA, prepared by partial DNAse I digestion of salmon sperm DNA according to Oleson and Koerner (1964). Salmon sperm DNA (Worthington) was dissolved to a final concentration of about 1 mg/mL in a 200 mL solution of 20 mM Tris-HCl [pH 7.4] and 1 mM EDTA. The addition of 10 μL of a fresh 1 mg/mL solution of DNAse I (Worthington) and $MgCl_2$ to a final concentration of 10 mM was followed by incubation at 30°C until about 15% of

the DNA was solubilized. After precipitation with 7% ice-cold trichloroacetic acid, the digested DNA was pelleted by centrifugation. The pellet was washed with 70% ethanol and recentrifuged. The activated DNA was dissolved to a final concentration of 10 mM in buffer containing 20 mM Tris-HCl [pH 8.6] and 0.1 mM EDTA and stored at -20°C.

Polymerase activity was assayed in 60 µL reactions that contained 67 mM Tris-HCl [pH 8.8], 16.7 mM $(NH_4)_2SO_4$, 0.5 mM DTT, 0.167 mM EDTA, 167 µg/mL bovine serum albumin, 83 µM dNTPs (one labeled [^{3}H]dNTP at 82 cpm/pmol), 833 µM activated DNA (expressed in nucleotide equivalents), and 3.3 nM T4 DNA polymerase. This mixture was incubated at 30°C for 5 min to allow the formation of a stable complex between the enzyme and DNA. The polymerase reaction was initiated by the addition of $MgCl_2$ to give a final concentration of 6.67 mM. Following further incubation for 5 and 10 min at 30°C, 20 µL samples of the reaction were stopped by spotting onto GF/A filters (Whatman) that had been prespotted with 20 µL of 0.1 M EDTA. The filters were floated in an ice-cold solution of 0.1 M $NaPP_i$ and 7.5% trichloroacetic acid. Following the collection of all samples, filters were washed under vacuum with 1% trichloroacetic acid and 95% ethanol, then dried and counted in scintillation fluor. One unit of DNA polymerase catalyzes the incorporation of 10 nmol of labeled nucleotide into an acid-insoluble product in 30 min at 30°C.

3'→5' Exonuclease Activity Assay

Exonuclease activity was measured on single-stranded DNA (Table II) and on a partially duplex primer-template formed by annealing the 20-mer primer to a 45-mer template (Table II) at a 1:2 ratio of primer to template. The 24 µL reaction mix was similar to the polymerase assay (67 mM Tris-HCl [pH 8.8], 16.7 mM $(NH_4)_2SO_4$, 0.5 mM DTT), but without dNTPs, and with either a single- or double-stranded DNA substrate (5.6 nM in terms of primer), and a 3-fold excess of T4 DNA polymerase (16.7 nM). After a pre-incubation of DNA polymerase and substrate for 5 min at 30°C, reactions were initiated by

Table II. DNA sequences of single- and double-stranded DNA substrates used to measure 3'→5' exonuclease activity.

DNA Substrate	DNA Sequence
Single-stranded	
20-mer	5'ATTACGAATGCCCACACCGC3'
Double-stranded	
20-mer (primer)	5'ATTACGAATGCCCACACCGC3'
45-mer (template)	3'TAATGCTTACGGGTGTGGCGGCCGCGGGTGGTGGTGGTGATCGACCG5'

The underline indicates that the 20-mer primer was 5'-end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase as described previously.

the addition of $MgCl_2$ to a final concentration of 6.67 mM. Reaction samples (6 µL), taken at 15 sec, 1 min, and 5 min, were stopped by the addition of 6 µL gel loading buffer (94 µL deionized formamide, 4 µL 10X Tris-borate-EDTA gel buffer, 2 µL 2% bromophenol blue/xylene cyanol blue). Samples were heated at 95°C for 5 min. Reaction products were separated by electrophoresis on a 15% polyacrylamide, 8 M urea gel. ^{32}P-labeled products were visualized by autoradiography with a phosphorimager (Molecular Dynamics).

Stopped-Flow Single-Turnover Excision Reactions

The 3'→5' exonuclease activity of mutant T4 DNA polymerases was measured on a millisecond time scale by following the release of the fluorescent nucleotide analogue, 2-aminopurine deoxyribonucleoside monophosphate (d2APMP), from the 3'-end of a DNA substrate. The free d2APMP nucleotide is highly fluorescent, but fluorescence is quenched when d2APMP is incorporated at the 3'-terminus of a DNA substrate.

The primer-templates used are described in Table III. Primer-templates contained either a 2-aminopurine-thymine (2AP-T) or 2-aminopurine-cytosine (2AP-C) terminal base pair and were either A+T- or G+C-rich in the region upstream of the 2AP base pair. The terminal 2AP-C mispair was present in three different contexts: no upstream mispairs, one upstream mispair, or two upstream mispairs. A T-C mispair was introduced immediately upstream of the 2AP-C mispair to produce a primer-template with two 3'-terminal mispairs. An additional upstream mispair (G-G in the G+C-rich primer template and A-G in the A+T-rich primer-template) was added upstream of the T-C and 2AP-C mispairs to give a primer-template with three terminal mispairs. The primer-templates were annealed in buffer containing 25 mM HEPES [pH 7.5] and 50 mM NaCl as previously described (Bloom *et al.*, 1994). Annealing mixtures were heated to 90°C and then slowly cooled to room temperature.

Table III. DNA sequences of 2AP-containing primer-templates.

Description	Name	DNA sequence
Primer 1[a] (G+C-rich)	XZ12	5' GCA CGT CAT TAA CGG TP[b] 3'
Template 1	XZ1	3' CGT GCA GTA ATT GCC ATG GAT CGA TGG TTT 5'
Template 2	XZ2	3' --- --- --- --- --- -C- --- --- --- --- 5'
Template 3	XZ6	3' --- --- --- --- --- CC- --- --- --- --- 5'
Template 4	XZ7	3' --- --- --- --- --G CC- --- --- --- --- 5'
Primer 2 (A+T-rich)	XZ13	5' GCA CGT CAT CGG TAA TP 3'
Template 1	XZ8	3' CGT GCA GTA GCC ATT ATG GAT CGA TGG TTT 5'
Template 2	XZ9	3' --- --- --- --- --- -C- --- --- --- --- 5'
Template 3	XZ10	3' --- --- --- --- --- CC- --- --- --- --- 5'
Template 4	XZ11	3' --- --- --- --- --G CC- --- --- --- --- 5'

[a]The oligonucleotides used to form the eight different primer-templates were generous gifts from L. Bloom. Dashes indicate sequence identical to XZ1 (G+C-rich) or XZ8 (A+T-rich).

[b]**P** designates the position of d2APMP at the 3'-terminus of the primers.

The rapid increase in fluorescence intensity caused by d2APMP release was measured using a SX.17 MV spectrofluorimeter (Applied Biophysics). Excision rates were measured in the stopped-flow apparatus using a 335 nm cut-off filter and 310 nm excitation light from a xenon arc source passing through a 2 mm band pass. The photomultiplier voltage setting was 700 volts and scattered light was subtracted from the reaction using an offset voltage. The reaction temperature was maintained at 20.0°C±0.5°C by allowing water from a thermostated bath to flow around the optical cell and reservoir syringes.

Reaction mixes for single-turnover excision experiments were prepared in two separate syringes. One syringe contained 1.4 μM T4 DNA polymerase, 2 mM DTT, and 16 mM $MgCl_2$,' in a buffer solution of 25 mM HEPES [pH 7.5] and 50 mM NaCl. The second syringe contained 400 nM single-stranded primer DNA or 400 nM primer-template DNA (expressed in terms of 3'-primer ends), and 0.5 mM EDTA in the above buffer. Reactions were initiated in the stopped-flow apparatus by mixing equal volumes (50 μL) of each solution within the instrument dead time of 1.5 ms by pneumatic rams triggered by 140 psi of N_2 gas. The final concentrations of reaction components were 700 nM T4 DNA polymerase, 200 nM DNA, 1 mM DTT, and 8 mM Mg^{2+}. Single turnover conditions were provided by using an excess of T4 DNA polymerase (700 nM) compared to the 200 nM DNA substrate. Excision reactions were also measured in the presence of dNTPs (Pharmacia) by adding dATP, dCTP, dGTP, and dTTP, each at a concentration of 200 μM to the enzyme-containing syringe. After mixing the contents of the two syringes, the final dNTP concentrations were 100 μM.

Excision rates were measured as a function of increased fluorescence (caused by the exonucleolytic release of d2APMP from the primer-terminus) with time. Fluorescence emission curves were fit to single (monophasic) or double (biphasic) exponential equations. Multiple runs (6 to 8) of each reaction were performed and the mean rate constants were calculated.

Temperature Sensitivity of the D131G-DNA Polymerase

The effect of elevated temperatures on the stability of the D131G mutant DNA polymerase was examined by heating the enzyme to various temperatures and then assaying for 5'→3' polymerase and 3'→5' exonuclease activities. Samples of wild type and D131G-DNA polymerase were incubated at 30°C, 35°C, 40°C, 45°C, and 50°C for 10 min and then placed on ice prior to being assayed for activity. Polymerase activity was assayed under the reaction conditions described above, except that EDTA was omitted from the reaction mix and reactions were enzyme-initiated. The exonuclease assay reaction mix was similar to that of the polymerase assay, except that dNTPs and EDTA were omitted from the reaction and the DNA substrate was 80 μM alkali-denatured *E. coli* [^{3}H]DNA (64 cpm/pmol/nucléotide). Reactions (60 μL) were initiated by the addition of enzyme (80 ng to give a final concentration of 13.3 nM) and were incubated at 30°C. After 6 and 12 min of incubation, 24 μL samples of the reaction were stopped by the addition of 0.38 mL of 0.5 mg/mL unlabeled single-stranded DNA, followed by the addition of 0.4 mL ice cold 15% trichloroacetic acid. Samples were chilled on ice for at least 10 min to precipitate the DNA. The substrate DNA was pelleted by centrifugation and 200 μL of the acid soluble supernatant was counted in scintillation fluor. One unit of exonuclease activity releases 10 nmol of acid soluble product in 30 min at 30°C.

RESULTS

The T4 DNA polymerase was selected as a model system to study the mechanism of exonucleolytic proofreading. Specifically, the contribution of D131 to the 3'→5' exonuclease activity of T4 DNA polymerase was addressed. Three amino acid substitutions, D131G, D131N, and D131S, were examined for their impact on proofreading. Characterization of the three mutant DNA polymerases included an assessment of DNA polymerase replication fidelity *in vivo* and biochemical assays on purified mutant enzymes to measure 5'→3' polymerase and 3'→5' exonuclease activities *in vitro*.

Isolation of T4 DNA Polymerase Mutator Mutants

The D131G-DNA polymerase mutator mutant was previously isolated by a powerful genetic selection procedure (Reha-Krantz *et al.*, 1986). Briefly, T4 phage with an amber nonsense mutation in the *rIIB* gene were randomly mutagenized and then plated under conditions restrictive for the *rII*$^-$ mutation. Only those phage which were able to significantly increase the reversion frequency of the *rII*$^-$ mutation could survive the selection. The D131N- and D131S-DNA polymerase mutants were isolated in similar genetic selections. The repeated isolation of mutator mutants with amino acid substitutions at codon 131 suggested the importance of D131 in maintaining replication fidelity, and therefore provided the basis of the present study.

Construction and In Vivo Characterization of Mutant T4 Phage Strains

The investigation into the role of D131 in proofreading was initiated by examining *in vivo* phenotypes of T4 phage strains with D131G-, D131N-, and D131S-mutant DNA polymerases. Because the original phage strains were mutagenized, and thus, may contain confounding mutations elsewhere in the genome, isogenic phage strains bearing mutations that encode the D131G, D131N, and D131S amino acid substitutions were constructed by

a marker rescue procedure (see Materials and Methods). The desired mutations were introduced into the T4 DNA polymerase expression vector (pTL7-g*43*) by site-directed mutagenesis. Bacteria carrying the mutagenized vector were then infected with a mutant T4 phage, Y128*am*, which has an amber nonsense mutation at codon 128 (in close proximity to D131). During the infection, homologous recombination between the phage gene *43* and the cloned copy of the gene on the vector produced recombinants in which the amber mutation region had been exchanged with the codon 131 region. Recombinant phage were selected based on loss of the amber phenotype. The DNA polymerase genes in the newly constructed D131G-, D131N-, and D131S-phage strains were sequenced to verify the presence of desired mutations and the absence of any confounding mutations. The *in vivo* phenotypes of the three mutant phage strains were compared to that of wild type T4 phage.

Mutant DNA polymerases can display a variety of phenotypes which include increased errors in DNA replication (mutator phenotype), decreased errors in DNA replication (antimutator phenotype), temperature sensitivity, and sensitivity to nucleotide and pyrophosphate analogues. Mutator and temperature sensitive phenotypes were detected for the mutant DNA polymerases previously (Reha-Krantz *et al.*, 1986; Reha-Krantz, 1988) and these phenotypes were re-examined for the newly constructed isogenic phage strains.

Two types of assay system, (1) a reversion assay or (2) a forward mutation assay, can be used to detect a mutator phenotype. In reversion assays, a marker serves as a reporter for the accuracy of DNA replication. In the study of mutant T4 phage, a commonly used reversion marker is a mutation in one of the *rII* genes. The frequency with which the *rII*$^-$ mutation *reverts* to *rII*$^+$ depends on the accuracy of DNA replication. Mutator DNA polymerases make more replication errors than the wild type DNA polymerase and consequently increase the reversion frequency of the *rII*$^-$ mutation. The more error-prone the mutant DNA polymerase is, the more *rII*$^-$ to *rII*$^+$ revertants will be produced.

Because mutations occur randomly, several phage lysates, each derived from a single phage particle, were prepared in order to determine the mutation frequency. The number of *rII*$^+$ revertants will be similar in most phage cultures, but a significantly higher or lower number may be measured in a few cultures. The culture with the median number of *rII*$^+$ revertants, therefore, provides an accurate measure of the mutation frequency. The reversion frequency values for reversion of the *rII* marker, *rIIUV199oc*, to *rII*$^+$ for wild type, D131S-, D131N-, and D131G-phage strains, and an exonuclease-deficient strain, D112A+E114A, are given in Table IV. The D131S- and D131N-DNA polymerases increased the spontaneous mutation frequency approximately 10-fold and the D131G-DNA polymerase increased the reversion frequency more than 200-fold, comparable to the exonuclease-deficient D112A+E114A-DNA polymerase.

The nature of reversion assays is such that the frequency of mutations at a *single* site is measured; hence, reversion frequency determinations are not representative of all of the mutational pathways. The *rIIUV199oc* mutation reverts primarily by one pathway, T→C transition in the TAA (*ochre*) codon (Reha-Krantz, 1995). Forward mutation assays, on the other hand, detect mutations at a variety of sites, and in many cases, by several mutational pathways. A convenient forward mutation assay for determining mutation frequencies in T4 phage is to measure the number of mutations that confer resistance to acriflavine. T-even bacteriophage are more sensitive than the host bacteria to acridine dyes, like acriflavine, because phage-induced changes in the bacterial surface increase the uptake of acriflavine from the medium (Silver, 1965). Mutations in the T4 *ac* gene reduce the uptake of acriflavine, thereby conferring resistance to the dye (Silver, 1965). The median numbers of acriflavine-resistant mutants for the wild type, D131S-, D131N-, D131G-, and D112A+E114A-phage strains are given in Table IV. Consistent with the mutator activity measured by the reversion assay, D131S- and D131N-DNA polymerases produced an approximate 10-fold increase in the frequency of mutations conferring resistance to acriflavine. The D131G-DNA polymerase, like the exonuclease-

Table IV. DNA replication fidelity of wild type and mutant T4 DNA polymerases.

	Mutator activity[a]	
Phage strains	*rIIUV199oc*$^+$	Acriflavine resistance
Wild type	1	1
D131S	10	5
D131N	15	10
D131G	250	85
D112A+E114A	300[b]	100[b]

[a]Increases in spontaneous mutation frequencies (mutator activity) were determined by measuring the number of *rIIUV199oc*$^+$ revertants and in a forward mutation assay that detects acriflavine-resistant mutants (see Materials and Methods). The values for mutator activity are given relative to wild type spontaneous mutation rates.

[b]Data are from Reha-Krantz and Nonay (1993).

deficient D112A+E114A-DNA polymerase, increased mutation frequency to acriflavine-resistance by about 100-fold.

A second phenotype, temperature sensitivity, was also examined. The newly constructed D131G-strain was temperature sensitive, based on the inability of this mutant strain to produce plaques at 42°C. Temperature sensitive phenotypes were not observed for the D131N- or D131S-mutant strains.

Purification of Mutant DNA Polymerases

Biochemical characterization of the D131S-, D131N-, and D131G-DNA polymerases required a source of purified enzymes. Site-directed mutagenesis was used to introduce mutations specifying the D131S, D131N, and D131G amino acid substitutions into the cloned T4 DNA polymerase gene. Transcripts produced by the mutated T4 DNA polymerase genes were sequenced to confirm the presence of desired mutations and the absence of additional mutations that may have arisen during site-directed mutagenesis. Mutant enzymes containing the D131S, D131N, or D131G amino acid substitutions were overproduced from bacterial cells containing the modified T4 DNA polymerase expression vectors. The mutant DNA polymerases were purified to near homogeneity using ion exchange chromatography, as previously described (see Materials and Methods). The wild type and D112A+E114A-DNA polymerases, which had been previously purified by an identical procedure, were included for comparison throughout the biochemical characterization of the D131S-, D131N-, and D131G-DNA polymerases.

DNA Polymerase Activity In Vitro

The first step in the biochemical characterization of mutant DNA polymerases was to determine if the amino acid substitutions affected DNA replication or proofreading activities. DNA polymerase activity was measured in reactions with activated DNA as the primer-template (see Materials and Methods). In the presence of dNTPs (one labeled with

a radioactive isotope) and Mg^{2+}, DNA polymerases incorporate dNMPs to produce radioactively-labeled DNA. An equal concentration of T4 DNA polymerase (3.3 nM) was used when assaying wild type and mutant enzymes. The rate of nucleotide incorporation relative to enzyme concentration gives the specific activity of the DNA polymerase. The specific activities for nucleotide incorporation by the wild type, D131S-, D131N-, and D131G-DNA polymerases were similar, but the exonuclease-deficient D112A+E114A-DNA polymerase was 10-fold less active (Table V).

Exonuclease Activity of T4 DNA Polymerase Mutants

Reduced exonuclease activity was predicted to be the cause of the mutator activities displayed by D131S-, D131N-, and D131G-mutant phage strains *in vivo* because D131 resides in the exonuclease domain of the T4 DNA polymerase. [Note position of D131 relative to exonuclease active site residues D112, D219, and D324 (Fig. 2)]. To test this hypothesis, the 3'→5' exonuclease activity of wild type and mutant DNA polymerases was examined on both single- and double-stranded DNA substrates. The single-stranded DNA substrate was a synthetic 20-mer oligonucleotide (Table II) that was 5'-end labeled with ^{32}P. The double-stranded DNA substrate was formed by annealing the labeled 20-mer to a synthetic 45-mer oligonucleotide (Table II). In the absence of dNTPs and in the presence of Mg^{2+}, the T4 DNA polymerase 3'→5' exonuclease activity will degrade the ^{32}P-labeled 20-mer strand to produce lower molecular weight products. The degradation products were separated by polyacrylamide gel electrophoresis on 15% acrylamide-8 M urea gels. The wild type and mutant enzymes were present at the same concentration (16.7 nM).

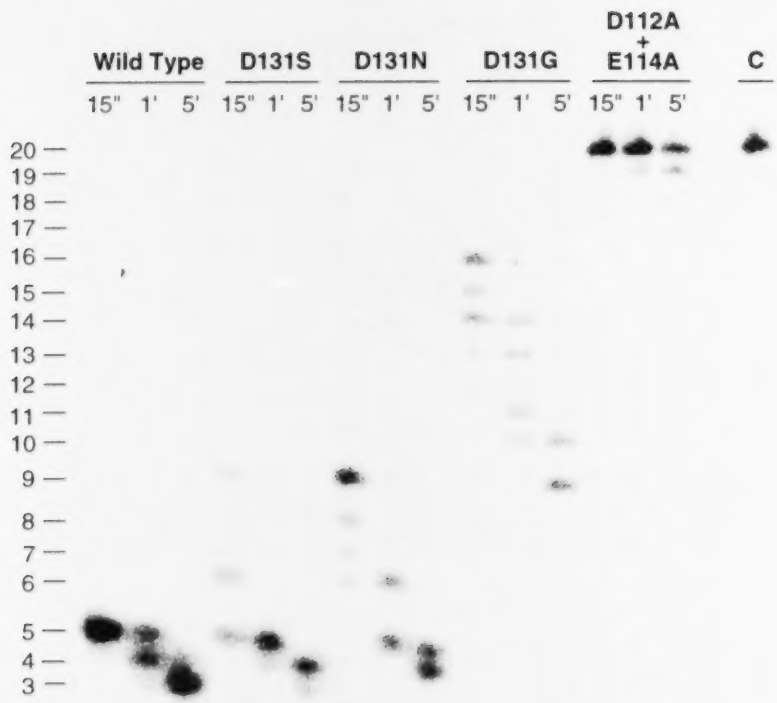
Wild type T4 DNA polymerase degraded the single-stranded 20-mer predominantly to the 5-mer in 15 s or less and degradation to the 3-mer occurred within 5 min (Fig. 3). The rapid degradation to the 5-mer indicates that degradation of oligonucleotides shorter than the 5-mer may proceed at a slower rate; possibly, slower enzyme association with the

Table V. DNA polymerase activity of wild type and mutant T4 DNA polymerases.

Enzyme	Polymerase activity[a]
	units/μg
Wild type	3.5
D131S	4.0
D131N	4.0
D131G	3.7
D112A+E114A	0.4

[a]DNA polymerase activity was measured using activated DNA as the primer-template (see Materials and Methods). One unit of DNA polymerase catalyzes the incorporation of 10 nmol of labeled nucleotide into an acid-insoluble product in 30 min at 30°C.

Figure 3. **Exonuclease activity of wild type and mutant T4 DNA polymerases on a single-stranded DNA substrate.** Wild type, D131S-, D131N-, D131G-, and D112A+E114A-DNA polymerases were incubated in reactions with a $5'^{32}$P-end labeled 20-mer oligonucleotide in the absence of dNTPs. Wild type or mutant enzymes were present in 3-fold excess (16.7 nM) over the single-stranded DNA substrate (5.6 nM). Reactions were initiated with the addition of $MgCl_2$ (C_f = 6.67 mM) and incubated at 30°C for 15 s, 1 min and 5 min reaction times. The control reaction (C) contained no T4 DNA polymerase.

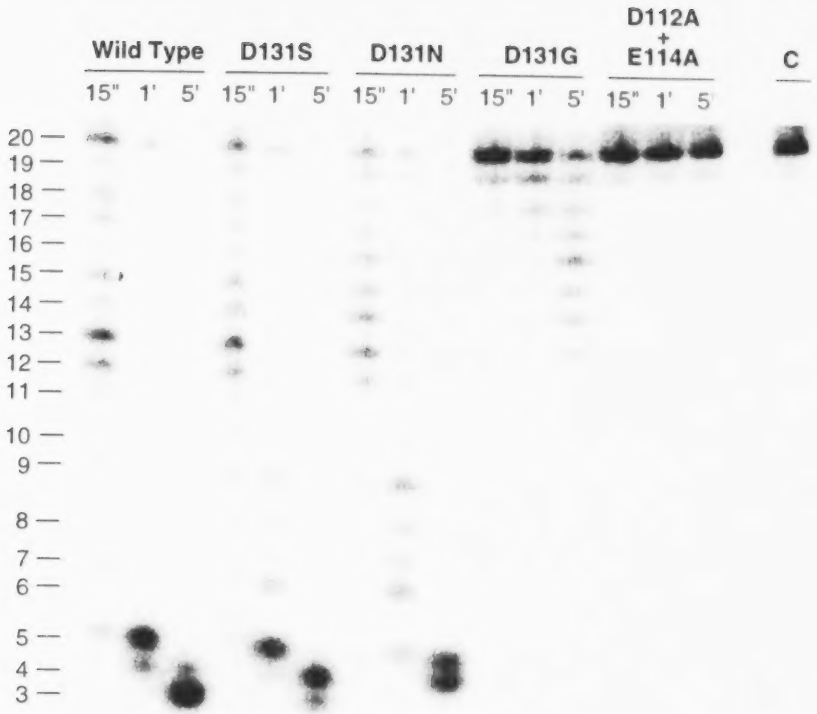

Wild Type
D131S
D131N
D131G
D112A + E114A
C
15" 1' 5' 15" 1' 5' 15" 1' 5' 15" 1' 5' 15" 1' 5'
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3

shorter DNA substrate compared to longer DNA substrates may limit the degradation process. Digestion of the single-stranded 20-mer by the mutant DNA polymerases revealed additional intermediates in the degradation process. Degradation products 9 and 6 nucleotides in length were observed in 15 s reactions with the D131S- and D131N-mutant DNA polymerases (Fig. 3). By 5 min, the D131S-DNA polymerase produced mostly the 4-mer, as well as a smaller amount of 3-mer. The 20-mer was converted to 5- and 4-mers by the D131N-DNA polymerase in 5 min, a level of degradation achieved in 1 min by the wild type enzyme (Fig. 3). Therefore, the D131S- and D131N-mutant DNA polymerases retain the ability to degrade single-stranded DNA, but at a slightly reduced rate compared to the wild type enzyme.

The exonuclease activity of the D131G-mutant DNA polymerase was substantially reduced compared to the wild type level on the single-stranded DNA substrate (Fig. 3). In a 15 s reaction, products ranging from 16- to 14-mers were observed, and by 5 min, further degradation occurred to produce 10- and 9-mers. This decreased level of exonuclease activity was still higher than the activity detected for the D112A+E114A-DNA polymerase, which showed little digestion even by 5 min (Fig. 3).

The natural DNA substrate for DNA polymerase exonuclease activity is duplex DNA with a mispaired primer-terminus, but the T4 DNA polymerase can also degrade fully duplex DNA in the absence of dNTPs. Thus, a double-stranded DNA substrate, compared to a single-stranded DNA substrate, more closely resembles the *in vivo* substrate for DNA polymerase-catalyzed proofreading. Exonucleolytic degradation of the 20-mer primer annealed to a 45-mer template (DNA substrates described in Table II) was slower than degradation of the single-stranded oligonucleotide (Fig. 4). Qualitatively, the level of degradation catalyzed by the wild type enzyme in 1 min with duplex DNA was similar to the amount degraded in 15 s with the single-stranded DNA substrate. Primarily 3-mer, along with a small proportion of 4-mer, were produced by the wild type enzyme by 5 min. As observed in reactions with single-stranded DNA, the degradation of the duplex DNA

Figure 4. **Exonuclease activity of wild type and mutant T4 DNA polymerases on a double-stranded DNA substrate.** Wild type, D131S-, D131N-, D131G-, and D112A+E114A-DNA polymerases were incubated in reactions with the 5'-^{32}P-end labeled 20-mer primer annealed to a 45-mer template. All enzymes were present in 3-fold excess (16.7 nM) over the double-stranded DNA substrate 5.6 nM in terms of primer). Reactions were initiated with the addition of $MgCl_2$ (C_f = 6.67 mM) and incubated at 30°C for 15 s, 1 min and 5 min reaction times. The control reaction (C) contained no T4 DNA polymerase.


Wild Type
D131S
D131N
D131G
D112A + E114A
C
15" 1' 5' 15" 1' 5' 15" 1' 5' 15" 1' 5' 15" 1' 5
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3

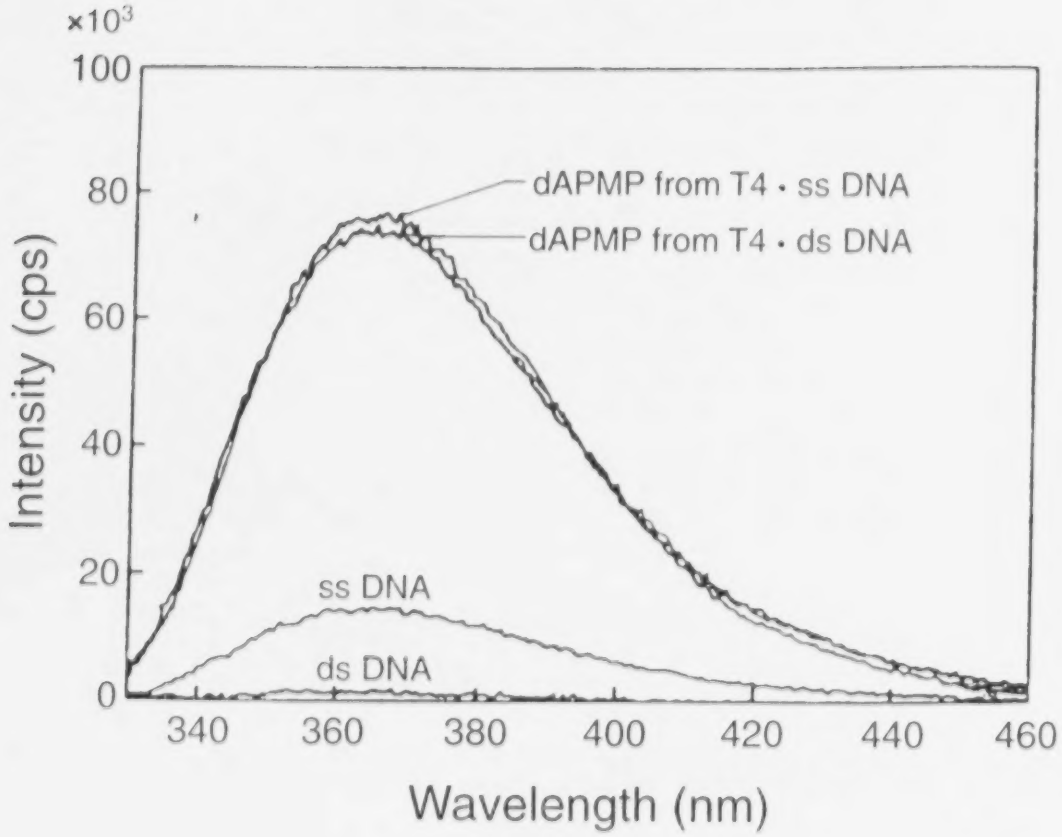
substrate by the D131S- and D131N-DNA polymerases was slightly slower than the rate measured for the wild type enzyme (Fig. 4). By 1 min, the D131S-DNA polymerase yielded primarily 5-mer, with some higher molecular weight products visible. By 5 min, the major degradation product of the D131S-DNA polymerase was a 4-mer, and some 3-mer was produced. Products ranged in size from 9- to 5-mers in a 1 min reaction with the D131N-DNA polymerase, and a mixture of 5- and 4-mers were observed by 5 min.

The exonuclease activity of the D131G-mutant DNA polymerase was very low on the double-stranded DNA substrate (Fig. 4). Only slight degradation occurred in 1 min and even after 5 min, high molecular weight species remained, ranging in length from 20- to 13-mers. As observed with the single-stranded DNA substrate, the D131G-DNA polymerase was more active than the D112A+E114A-DNA polymerase (Fig. 4).

Pre-Steady State Kinetics of 3'→5' Exonuclease Activity

The 3'→5' exonuclease activities of the mutant enzymes were further evaluated by a sensitive assay that employs rapid-mixing, stopped-flow techniques to detect the release of the fluorescent nucleotide analogue, d2APMP, from a primer-terminus within the millisecond time scale. The fluorescence of d2APMP in DNA (2AP-DNA) is quenched but the free d2APMP nucleotide is highly fluorescent (Fig. 5). The difference in fluorescence between d2APMP incorporated at the 3'-terminus of an oligonucleotide compared to free d2APMP provides the basis of the assay. The excision reactions were initiated in a stopped-flow apparatus by mixing a solution of enzyme and Mg^{2+} with a solution containing the DNA substrate, described in Table III. Two types of DNA substrate were used; one was relatively more G+C-rich near the terminal 2AP-T base pair and the second was relatively more A+T-rich. High A+T-richness near the primer-terminus is expected to increase excision rates by facilitating local melting of the DNA strands (Bessman and Reha-Krantz, 1977; Carver *et al.*, 1994).

Figure 5. **Fluorescence of free d2APMP and 2AP-DNA.** Fluorescence emission spectra for d2APMP incorporated at the 3'-terminus of single- and double-stranded DNA and as a free nucleotide. (Reproduced with permission from Bloom *et al.*, 1994).

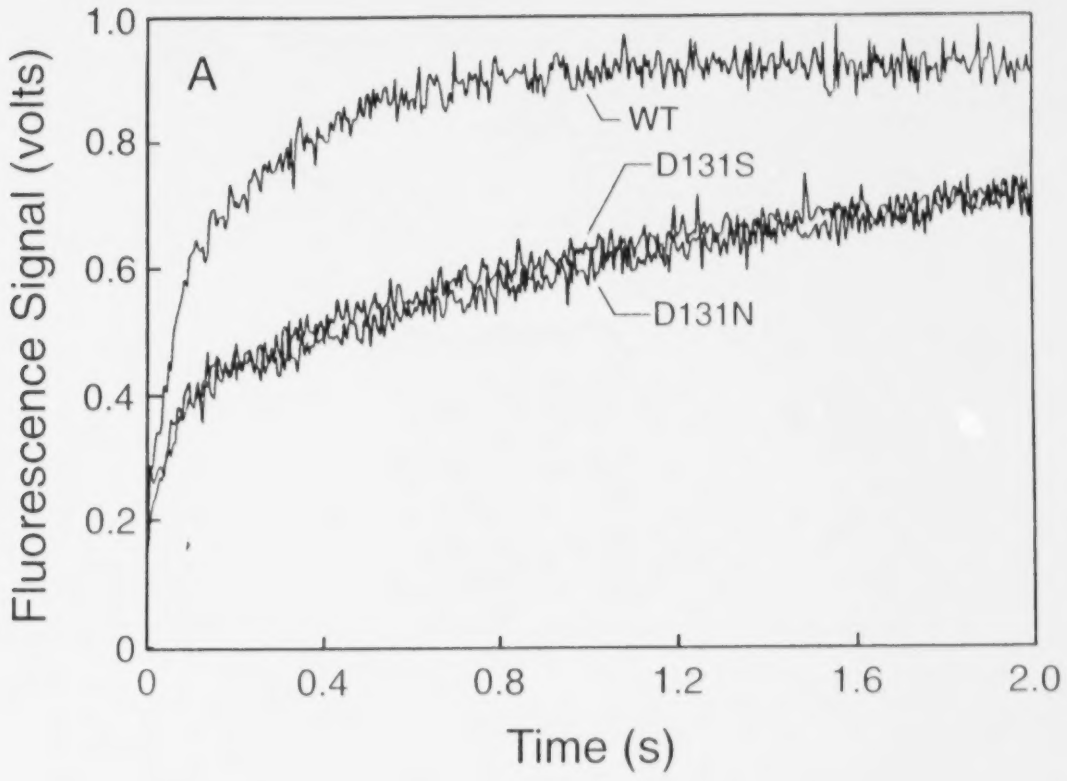

×10³
100
80
60
40
20
0
Intensity (cps)
dAPMP from T4 • ss DNA
dAPMP from T4 • ds DNA
ss DNA
ds DNA
340
360
380
400
420
440
460
Wavelength (nm)

The kinetic traces for the release of d2APMP from a 3'-terminal 2AP-T base pair in a G+C-rich DNA substrate (Table III) by wild type and mutant T4 DNA polymerases are shown in Figure 6. The fastest excision rate was observed for wild type T4 DNA polymerase (Fig. 6A). Slower excision rates were observed for the D131S- and D131N-DNA polymerases (Fig. 6A) and much slower rates were detected for the D131G- and D112A+E114A-DNA polymerases (Fig. 6B).

The reactions with the wild type, D131S-, and D131N-mutant DNA polymerases appeared to be biphasic. The kinetic traces for the three enzymes (Fig. 6A) were fit to both single- and double-exponential equations. The curve fits and residuals for both single-exponential (Fig. 7A) and double-exponential (Fig. 7B) equations for the wild type reaction show how well the two types of equation fit the data. The experimental data were better fit by the curve of a double exponential equation (Fig. 7A) than the curve of a single exponential equation (Fig. 7B), which was not superimposed on the experimental trace for early reaction times. The residuals given below each curve fit show the deviation of the experimental data from the curve described by the equation. A more uniform distribution of the residuals at early reaction times was observed for the double- compared to the single-exponential fit, which indicates that the double-exponential equation provides a better description for the data. The biphasic nature of the hydrolysis reactions with wild type, D131S-, and D131N-DNA polymerases implies the existence of two rate-determining reaction steps.

The pre-steady state excision rates calculated from the biphasic kinetic traces shown in Figure 6B are given in Table VI. The two reaction rates, k_1 and k_2, describe the respective fast and slow phases of biphasic reactions, with the relative amplitude of each phase reflecting the contribution of that phase to the overall reaction rate. Excision rates were also measured for the release of d2APMP from a terminal 2AP-T base pair in an A+T-rich primer-template. The biphasic kinetics observed for wild type, D131S-, and D131N-DNA polymerases with the G+C-rich primer-template were also observed with the

Figure 6. **Excision of d2APMP from a 3'-terminal 2AP-T base pair by wild type and mutant T4 DNA polymerases.** (A) Time courses for the removal of d2APMP by wild type, D131S-, and D131N-DNA polymerases with the G+C-rich primer-template containing a 3'-terminal 2AP-T base pair (XZ12/XZ1, Table III). Excision reactions were initiated by rapid mixing of a solution of DNA polymerase and Mg^{2+} with a second solution containing the DNA substrate. Final concentrations of reaction components were the following: 700 nM T4 DNA polymerase, 200 nM DNA, 8 mM Mg^{2+} and 1 mM DTT. Reactions were allowed to proceed for 2 s at room temperature, 20°C. (B) Time courses for D131G- and D112A+E114A-DNA polymerases with the same 2AP-T primer-template. Reactions were as described above, except that the incubation time was extended to 200 s.


A
WT
D131S
D131N
Fluorescence Signal (volts)
Time (s)
0
0.2
0.4
0.6
0.8
1.0
0.4
0.8
1.2
1.6
2.0

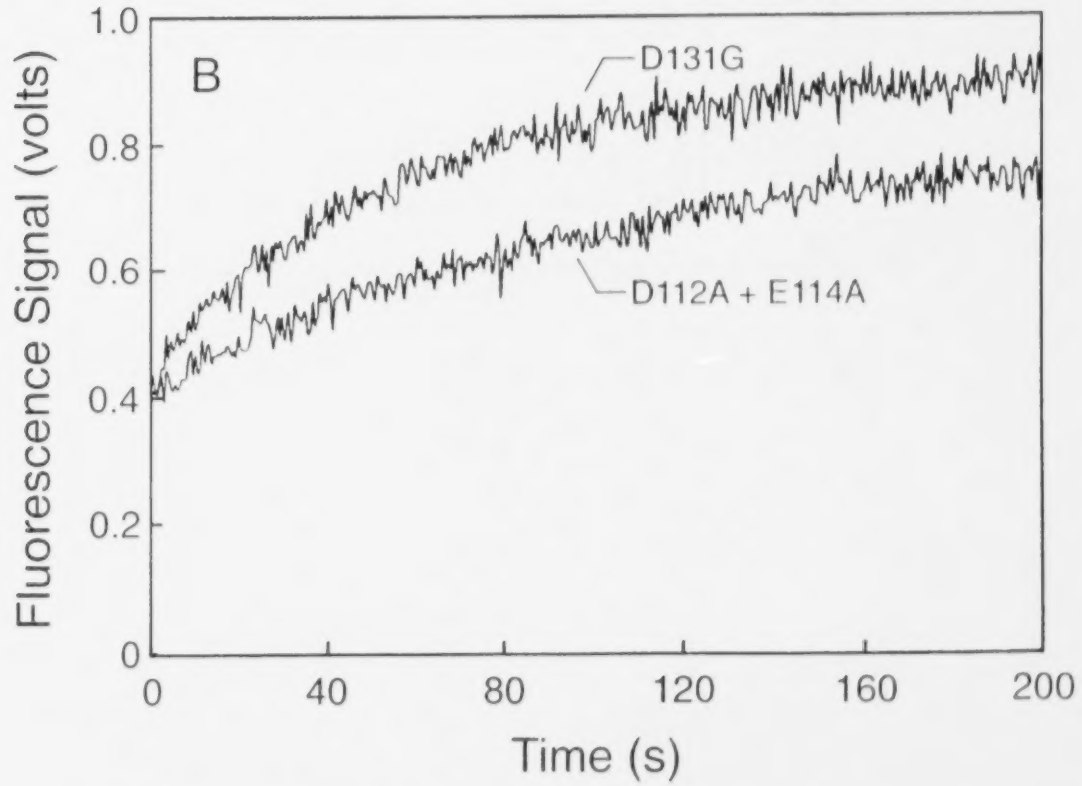
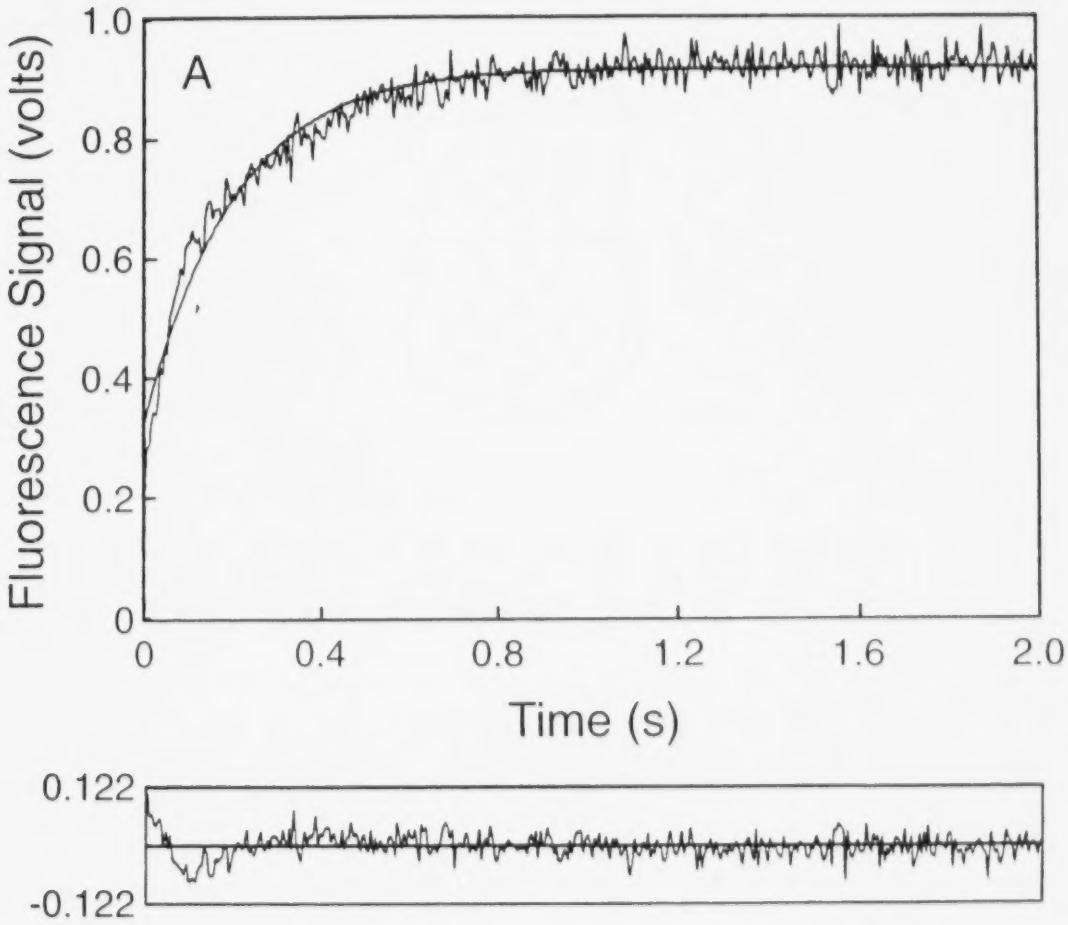

B
D131G
D112A + E114A
Fluorescence Signal (volts)
Time (s)
0
0.2
0.4
0.6
0.8
1.0
40
80
120
160
200

Figure 7. **Biphasic excision of d2APMP from a 3'-terminal 2AP-T base pair by the wild type T4 DNA polymerase.** The wild type excision reaction (Fig.6A) was fit to (A) single-exponential and (B) double exponential equations. The curve fits for excision reactions with the D131S- and D131N-DNA polymerases (not shown) are also better described by a double-exponential equation.


A
Fluorescence Signal (volts)
1.0
0.8
0.6
0.4
0.2
0
0
0.4
0.8
1.2
1.6
2.0
Time (s)
0.122
-0.122

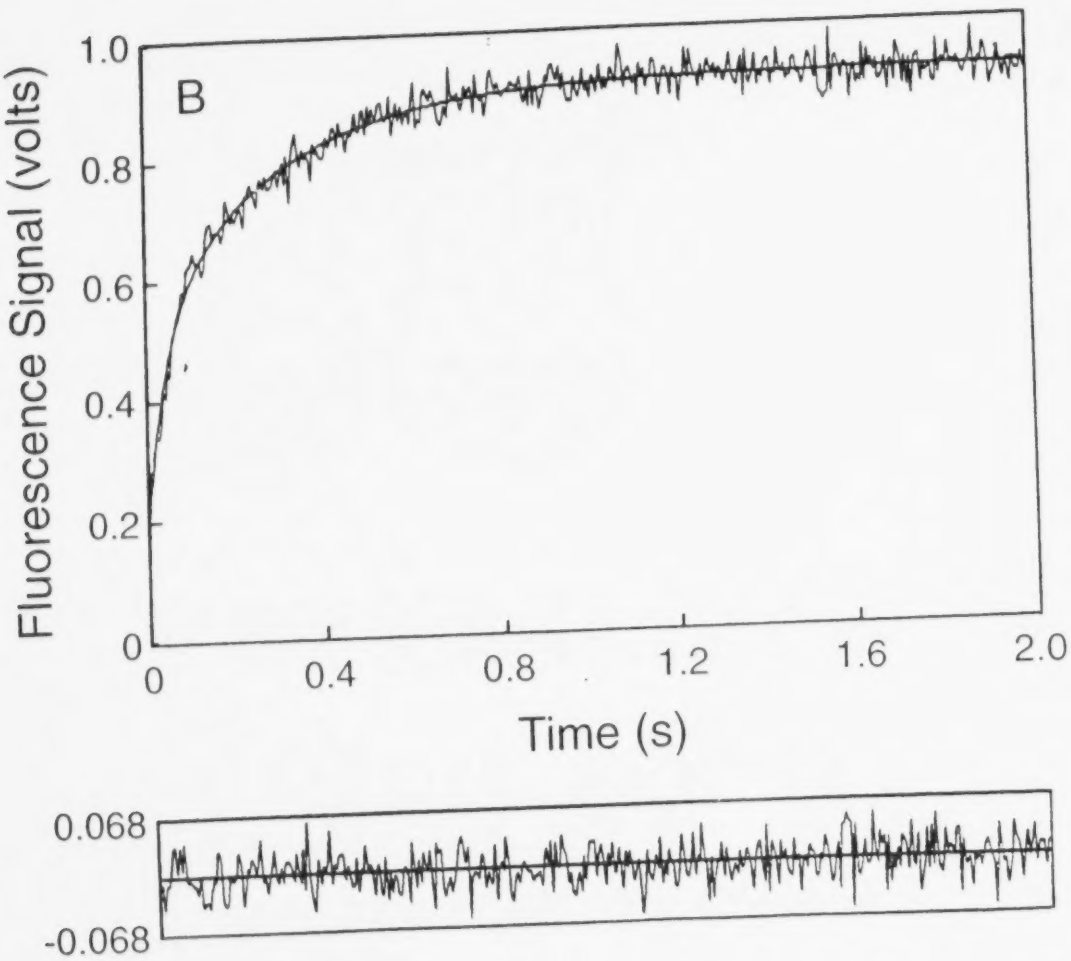

B
Fluorescence Signal (volts)
1.0
0.8
0.6
0.4
0.2
0
0
0.4
0.8
1.2
1.6
2.0
Time (s)
0.068
-0.068

Table VI. Pre-steady state excision rates of T4 DNA polymerases on double-stranded 2AP-T primer-templates.

	G+C-rich DNA substrate				A+T-rich DNA substrate			
Enzyme	$k_1(s^{-1})$[a]	a_1[b]	$k_2(s^{-1})$	a_2	$k_1(s^{-1})$	a_1	$k_2(s^{-1})$	a_2
Wild type	20.1 ± 2.7	(0.4)	3.6 ± 0.2	(0.6)	43.0 ± 3.8	(0.3)	11.9 ± 0.8	(0.7)
D131S	20.1 ± 1.1	(0.3)	1.0 ± 0.1	(0.7)	35.4 ± 2.5	(0.4)	1.4 ± 0.1	(0.6)
D131N	21.6 ± 2.9	(0.4)	0.5 ± 0.1	(0.6)	36.8 ± 4.4	(0.3)	0.7 ± 0.1	(0.7)
D131G[c]			0.019 ± 0.001				0.024 ± 0.001	
D112A+E114A[c]			0.010 ± 0.002				0.015 ± 0.005	

[a]The rate constants for the two phases of biphasic excision reactions are given by k_1 and k_2, respectively.

[b]The amplitudes of the two phases are designated a_1 and a_2 , respectively.

[c]The rate constants for the monophasic excision reactions with the D131G- and D112A+E114A-DNA polymerases are given in the k_2 column for comparison to the slower of the two rates detected for the wild type, D131S-, and D131N-DNA polymerases. It cannot be assumed, however, that the reaction step that is rate-limiting for the D131G- and D112A+E114A-DNA polymerases is the same rate-limiting reaction step as that described by the rateconstant k_2.

A+T-rich primer-template. For the wild type enzyme, the rates of each phase were at least 2-fold higher on the A+T- compared to the G+C-rich substrate (Table VI). Because A+T-richness lowers the melting temperature of DNA, the higher rates detected for the wild type enzyme with the A+T-rich primer-template can be explained by an increased ability of the DNA polymerase to separate the strands of an A+T-rich primer-template in preparation for hydrolysis.

The D131S- and D131N-DNA polymerases differed from the wild type enzyme in the slow phase of the biphasic reaction, described by rate constant k_2. With the G+C-rich DNA substrate, the k_2 rates were 3- to 6-fold lower than wild type and with the A+T-rich substrate, the rates were about 10- to 20-fold lower than detected for the wild type enzyme. In contrast to the wild type DNA polymerase, excision rates described by k_2 were not significantly stimulated by adjacent A-T base pairs.

The kinetic traces for the D131G- and D112A+E114A-mutant DNA polymerases (Fig. 6A) were fit to single exponential equations. Excision of d2APMP by the D131G-DNA polymerase was approximately 200-fold slower than the wild type excision rate on the G+C-rich primer-template and 500-fold slower on the A+T-rich DNA substrate (Table VI). This substantial decrease in exonuclease activity is consistent with the high mutator activity observed for the D131G mutant *in vivo* (Table IV). Even less exonuclease activity was detected for the exonuclease-deficient D112A+E114A-DNA polymerase, with excision rates reduced by 400- and 800-fold compared to the wild type enzyme on the G+C- and A+T-rich primer-templates, respectively (Table VI).

Excision of d2APMP from a Single-Stranded DNA Substrate

In order to confirm that mutations at D131 decrease exonuclease activity on both single- and double-stranded DNA substrates, the excision rates for wild type and D131G-DNA polymerases were compared using the 2AP-terminated oligonucleotides (XZ12 and XZ13, Table III). The wild type excision rates on G+C- and A+T-rich single-stranded

DNA substrates were in the range of 200 s^{-1} (Table VII). These rates are much higher than those observed for double-stranded DNA because, unlike a double-stranded primer-terminus which must be partially melted prior to hydrolysis, single-stranded DNA can bind directly in the exonuclease active center and undergo immediate hydrolysis. The D131G-DNA polymerase excised d2APMP from G+C- and A+T-rich single-stranded DNA substrates at rates approximately 100- to 200-fold lower than the wild type excision rates (Table VII). These results are consistent with the reduced exonuclease activity observed for the D131G-DNA polymerase on the radioactively-labeled single-stranded DNA substrate (Fig. 3). The decreased ability of D131G to hydrolyze both single- and double-stranded DNA suggests that this mutant is defective in a reaction step that is required for exonuclease activity on both types of DNA substrate. The substantially reduced exonuclease activity of the D131G-DNA polymerase implies that this mutant enzyme may be defective in the chemical step of the hydrolysis reaction. The D131N- and D131S-DNA polymerases, however, have significant levels of residual exonuclease activity; thus, further characterization of these mutant enzymes may implicate D131 in a rate-limiting step of the proofreading reaction that occurs before hydrolysis.

Effect of Increasing Strand Separation on Excision Rates

The involvement of D131 in strand separation was investigated by measuring the rate of d2APMP excision from DNA substrates with an increasing number of 3'-terminal mispairs by the D131N-DNA polymerase (Table VIII). The data for the wild type enzyme and the G255S-DNA polymerase, which has an apparent defect in strand separation (Marquez and Reha-Krantz, 1996), with the same primer-templates are included for comparison (Tables IX and X). Increasing the number of terminal mispairs from one to three simulates increasing strand separation, such that wild type excision rates on DNA substrates with three terminal mispairs (Table IX) approach excision rates measured with single-stranded DNA (Table VII). Increased A+T-richness near the primer-terminus,

Table VII. Pre-steady state excision rates of T4 DNA polymerases on single-stranded 2AP-DNA substrates.

Enzyme	G+C-rich DNA substrate	A+T-rich DNA substrate
	$k_1(s^{-1})$[a]	$k_1(s^{-1})$
Wild type	175.7 ± 5.7	228.4 ± 12.4
D131G	1.80 ± 0.05	0.99 ± 0.03

[a]The single rate constant, k_1, describes the monophasic excision of d2APMP from a single-stranded DNA substrate.

Table VIII. Pre-steady state excision rates of the D131N-DNA polymerase on DNA substrates with increasing pre-formed strand separation.

				G+C-rich DNA substrates				A+T-rich DNA substrates			
Number of mispairs	Description of 3'-mispairs[a]			$k_1(s^{-1})$[b]	a_1[c]	$k_2(s^{-1})$	a_2	$k_1(s^{-1})$	a_1	$k_2(s^{-1})$	a_2
0			2AP-T	21.6 ± 2.9	(0.4)	0.5 ± 0.1	(0.6)	36.8 ± 4.4	(0.3)	0.7 ± 0.1	(0.7)
1			2AP-C	18.5 ± 1.7	(0.5)	1.4 ± 0.1	(0.5)	40.0 ± 2.8	(0.6)	1.7 ± 0.1	(0.4)
2		T-C	2AP-C	27.2 ± 1.3	(0.7)	3.9 ± 0.3	(0.3)	113.7 ± 8.4	(0.5)	2.3 ± 0.6	(0.5)
3	A/G-G	T-C	2AP-C	155.5 ± 14.2				253.6 ± 22.8			

[a]The complete sequences of the DNA substrates are described in Table III.

[b]The rate constants for the two phases of biphasic excision reactions are given by k_1 and k_2, respectively.

[c]The amplitudes of the two phases are designated a_1 and a_2, respectively.

Table IX. Pre-steady state excision rates of wild type T4 DNA polymerase on DNA substrates with increasing pre-formed strand separation.

		G+C-rich DNA substrates				A+T-rich DNA substrates			
Number of mispairs	Description of 3'-mispairs[b]	$k_1(s^{-1})$[c]	a_1[d]	$k_2(s^{-1})$	a_2	$k_1(s^{-1})$	a_1	$k_2(s^{-1})$	a_2
0	2AP-T	20.1 ± 2.7	(0.4)	3.6 ± 0.2	(0.6)	43.0 ± 3.8	(0.3)	11.9 ± 0.8	(0.7)
1[a]	2AP-C	19.9 ± 0.3				42.0 ± 2.1			
2	T-C 2AP-C	87.8 ± 1.4				156.5 ± 7.1			
3	A/G-G T-C 2AP-C	157.2 ± 3.8				202.2 ± 12.4			

[a]Data for DNA substrates with one, two, and three terminal mispairs are from Marquez and Reha-Krantz (1996).

[b]The complete sequences of the DNA substrates are described in Table III.

[c]The rate constants for the two phases of biphasic excision reactions are given by k_1 and k_2, respectively.

[d]The amplitudes of the two phases are designated a_1 and a_2, respectively.

Table X. Pre-steady state excision rates of the G255S DNA polymerase[a] on DNA substrates with increasing pre-formed strand separation.

		G+C-rich DNA substrates				A+T-rich DNA substrates			
Number of mispairs	Description of 3'-mispairs[b]	$k_1(s^{-1})$[c]	a_1[d]	$k_2(s^{-1})$	a_2	$k_1(s^{-1})$	a_1	$k_2(s^{-1})$	a_2
0	2AP-T	14.9 ± 1.8	(0.4)	0.34 ± 0.01	(0.6)	19.2 ± 1.4	(0.4)	0.42 ± 0.02	(0.6)
1	2AP-C	12.6 ± 3.0	(0.3)	1.7 ± 0.1	(0.7)	19.0 ± 3.7	(0.5)	1.5 ± 0.3	(0.5)
2	T-C 2AP-C	52.4 ± 2.2				65.4 ± 8.0			
3	A/G-G T-C 2AP-C	62.4 ± 3.6				69.7 ± 2.0			

[a]Data are from Marquez and Reha-Krantz (1996).

[b]The complete sequences of the DNA substrates are described in Table III.

[c]The rate constants for the two phases of biphasic excision reactions are given by k_1 and k_2, respectively.

[d]The amplitudes of the two phases are designated a_1 and a_2, respectively.

which increases local melting, also stimulates wild type excision rates by approximately 2-fold (Table IX).

The excision rates for the mutant D131N- and G255S-DNA polymerases were also stimulated by increasing pre-formed strand separation (Tables VIII and X). The most significant difference between the wild type and mutant enzymes was that monophasic excision reactions were observed for the wild type enzyme, while the D131N- and G255S-DNA polymerases showed biphasic reaction kinetics on the 2AP-C DNA substrates (Tables VIII, IX, and X). The D131N-DNA polymerase excision reactions even remained biphasic on DNA substrates with two terminal mispairs (Table VIII). The rates of the slow phase (k_2) for both the D131N- and G255S-DNA polymerases were decreased by approximately 10- to 30-fold compared to the rates detected for the wild type enzyme on the 2AP-T DNA substrates. The D131N- and G255S-DNA polymerases also showed similar k_2 rates on the 2AP-C DNA substrates. Unlike the wild type k_2 rate, which was stimulated by increased A+T-richness, the k_2 rates for the D131N- and G255S-DNA polymerases were not significantly higher on the more A+T-rich DNA substrates.

The rates of the fast phase (k_1) for the D131N-DNA polymerase were more comparable to the wild type k_1 rates than to those of the G255S-DNA polymerase. The k_1 rates for the wild type and D131N-DNA polymerases were increased by about 2-fold on A+T-rich compared to G+C-rich DNA substrates, while k_1 rates for the G255S-DNA polymerase were relatively unaffected by increased A+T-richness. A significant increase in the k_1 rate for the D131N-DNA polymerase was observed on the G+C-rich DNA substrate with three terminal mispairs and on the A+T-rich DNA substrate with two terminal mispairs. Since A+T-rich primer-termini are more prone to melting, the A+T-rich DNA substrate with two terminal mispairs likely has a similar degree of pre-formed strand separation as the G+C-rich DNA substrate with three terminal mispairs. Therefore, three terminal mispairs appear to be required to significantly stimulate excision rates for the D131N-DNA polymerase. The k_1 rates for the G255S-DNA polymerase are still lower

than those of the wild type and D131N-DNA polymerases, even on DNA substrates with three terminal mispairs. The G255S-DNA polymerase, however, has wild type levels of exonuclease activity on single-stranded DNA substrates, which suggests that the defect of this mutant enzyme can be overcome if sufficient pre-formed strand separation is provided. For this reason, the G255S-DNA polymerase is proposed to be defective in strand separation (Marquez and Reha-Krantz, 1996). Wild type excision rates were measured for the D131N-DNA polymerase on DNA substrates with three terminal mispairs, which implies that the D131N-DNA polymerase is also defective in some aspect of strand separation.

Competition Between Extension and Excision Reactions

The 2AP-fluorescence assay was modified by the addition of dNTPs to simulate *in vivo* DNA polymerase replication conditions in which proofreading must compete with primer elongation. Correctly base paired primer-termini are readily extended, while mispaired primer-termini are extended more slowly (reviewed by Goodman *et al.*, 1993). The reduced rate of extension for a mispaired primer-terminus provides opportunity for the primer-terminus to be transferred to the exonuclease active center where the incorrect nucleotide is removed. The 2AP-T terminal base pair, while more correct than a base pair between 2AP and any of the other three bases is, nevertheless, recognized by DNA polymerases as less stable than the natural A-T base pair (Eritja *et al.*, 1986). Hence, the 2AP-T primer template resembles the natural substrate for proofreading, duplex DNA with a 3'-terminal mispair.

In order for the extension reaction to compete with excision, the 2AP-T primer-terminus must be capable of being bound in the polymerase active center ($Enzyme^{pol}$:2AP-T DNA). A partitioning of the 2AP-T DNA between polymerase and exonuclease active centers is consistent with the biphasic kinetics observed in the excision experiments. The fast phase could be attributed to the rapid excision of d2APMP from a primer-terminus

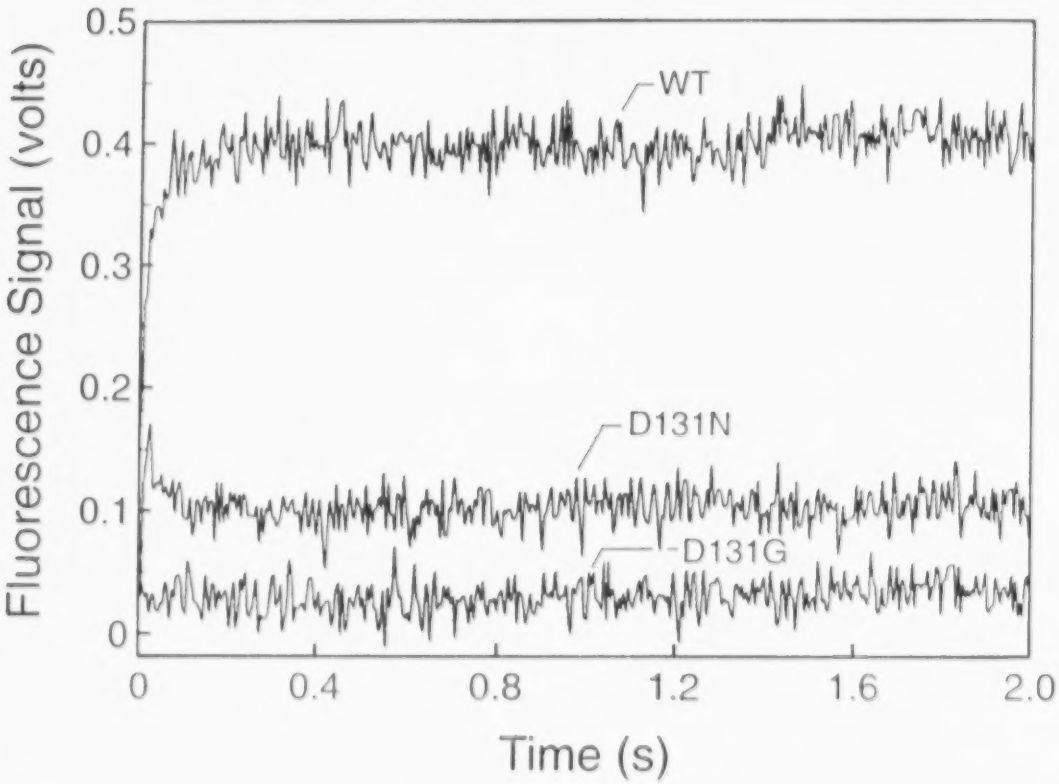
bound in the exonuclease active center, while the transfer of the primer-terminus from the polymerase to exonuclease active center ($Enzyme_{exo}$:2AP-T DNA) could produce the slow phase of the excision reaction. This hypothesis was tested by determining if the 2AP-T primer-terminus could be extended in the presence of dNTPs, since extension requires the formation of an $Enzyme^{pol}$:2AP-T DNA complex.

Extension:excision competition experiments were initiated in the stopped-flow apparatus by the rapid mixing of a solution containing enzyme, Mg^{2+}, and dNTPs with a solution containing the DNA substrate. If the primer-terminus is bound in the polymerase active center in a state activated for nucleotide incorporation, replication will proceed, thereby precluding the excision reaction. Thus, extension of the 2AP-T "mispair" in the presence of dNTPs will result in a smaller amount of fluorescent d2APMP released from the $Enzyme^{pol}$:2AP-T DNA complex compared to reactions performed in the absence of dNTPs. The extent to which d2APMP production is decreased in the presence of dNTPs will reflect the proportion of 2AP-T primer-termini extended.

The kinetic traces for competition experiments between extension and excision reactions with wild type, D131N-, and D131G-DNA polymerases are shown in Figure 8. The amount of d2APMP released by the wild type enzyme in the presence of dNTPs (Fig. 8) was only about one half of the amount produced in the absence of dNTPs (Fig. 6A), indicating that about half of the 2AP-primers were extended (Fig. 8). Based on an amplitude (a_2) of about 0.6 for the slow phase of the wild type excision reaction in the absence of dNTPs, approximately 60% of primer-termini were proposed to be bound in the polymerase active center. Thus, the majority of these primer-termini were extended in the presence of dNTPs to account for the 50% decrease in d2APMP excision observed. These results provide support for the existence of an $Enzyme^{pol}$:2AP DNA complex.

A small burst of d2APMP release was observed for the D131N- and D131S-DNA polymerases, but the fluorescence intensity remained at a low level for the duration of the

Figure 8. **Excision of d2APMP from a 3'-terminal 2AP-T base pair in the presence of deoxyribonucleoside triphosphates.** Time courses for the excision reactions with wild type, D131N-, and D131G-DNA polymerases with the A+T-rich, 2AP-T primer-template in the presence of 100 mM dNTPs. The D131S-DNA polymerase time course (not shown) was identical to that of the D131N-DNA polymerase.


Fluorescence Signal (volts)
0.5
0.4
0.3
0.2
0.1
0
WT
D131N
D131G
0
0.4
0.8
1.2
1.6
2.0
Time (s)

reaction. The reduced hydrolysis of d2APMP from a 2AP-T base pair in the presence of dNTPs reflects the greater ability of these mutant enzymes to extend the 2AP-T primer-terminus and may account for the 10-fold increase in spontaneous mutation rates observed for the D131N- and D131S-DNA polymerase mutants *in vivo*. In extension:excision competition reactions with the D131G-DNA polymerase, fluorescence levels did not rise above the level of 2AP-DNA, even by 200 s. The complete failure to excise d2APMP in the presence of dNTPs suggests that this mutant is even more likely to extend the 2AP-T base pair than the D131N- and D131S-DNA polymerases. This observation is consistent with the higher mutator activity of the D131G-DNA polymerase mutant compared to the D131N- and D131S-DNA polymerase mutants *in vivo*.

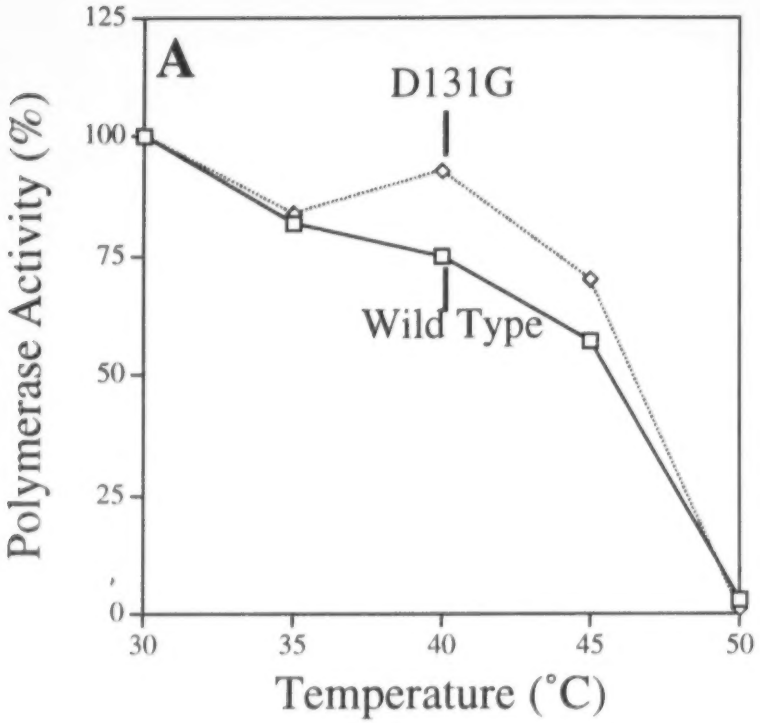
Temperature Sensitivity of the D131G-DNA Polymerase

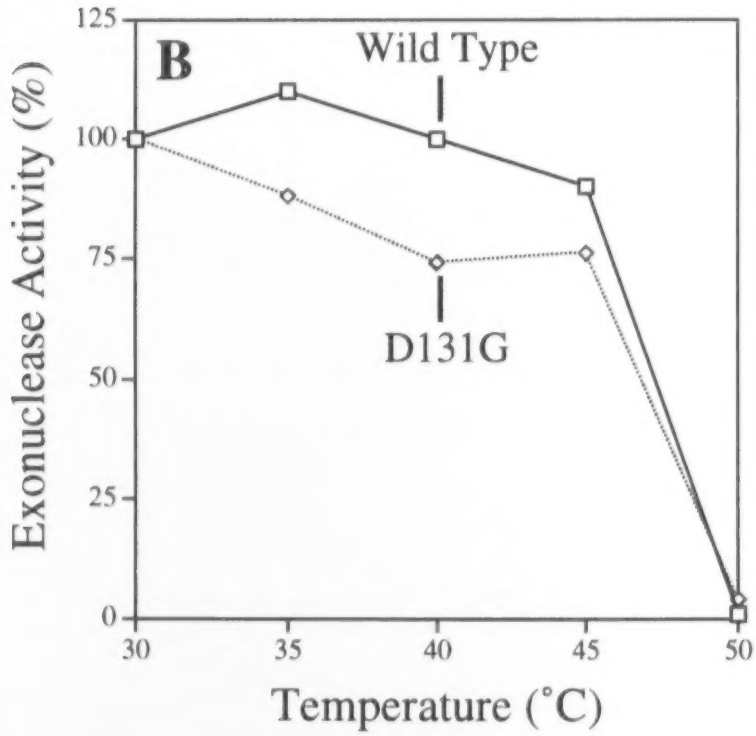
The temperature sensitivity of the D131G-DNA polymerase was examined *in vitro*. Although the D131G-DNA polymerase mutant has a temperature sensitive phenotype *in vivo* (Reha-Krantz *et al.*, 1986), the activity of the mutant enzyme may not be sensitive to high temperatures since temperature sensitivity can also arise due to a defect in folding the DNA polymerase into its tertiary form (Gordon and King, 1994). If the D131G-DNA polymerase is temperature sensitive for folding, the purified enzyme, which is already folded, should not display sensitivity to elevated temperatures. If, however, the exonuclease active center in the D131G-DNA polymerase is denatured at high temperatures, the exonuclease activity will decline as the temperature is increased.

Polymerase and exonuclease activity assays were performed at 30°C on samples of wild type and D131G-DNA polymerases that had been pre-incubated for 10 min at 30°C, 35°C, 40°C, 45°C, or 50°C prior to being assayed. DNA polymerase activity was measured using activated DNA as the primer-template as described previously, except that reactions were enzyme-initiated. Exonuclease activity was determined from the release of radioactively-labeled nucleotides from 80 μM *E. coli* [^{3}H]DNA in a reaction with 13.3 nM

enzyme. Heat inactivation of the polymerase activities of the wild type and D131G-DNA polymerases followed a similar trend, with complete inactivation observed at 50°C (Fig. 9A). Exonuclease activity of the wild type enzyme and the residual exonuclease activity of the D131G-DNA polymerase also followed similar patterns of heat inactivation (Fig. 9B). These results suggest that the D131G-mutant DNA polymerase exonuclease activity is not inherently more temperature-sensitive than the wild type T4 DNA polymerase. Thus, the temperature sensitivity of the D131G-DNA polymerase mutant *in vivo* can probably be attributed to defective folding of the mutant DNA polymerase at high temperatures.

Figure 9. **Decrease in polymerase and exonuclease activities caused by heat-inactivation of wild type and D131G-DNA polymerases.** Wild type and D131G-DNA polymerases were incubated for 10 min at 30°C, 35°C, 40°C, 45°C, and 50°C and then chilled on ice. Samples of the heat-treated enzymes were used to initiate polymerase and exonuclease assays that were incubated at 30°C. (A) Polymerase activity was assayed on 833 μM activated DNA in a reaction mixture containing 83 μM dNTPs (one labeled [^{3}H]dNTP at 82 cpm/pmol) and 3.3 nM T4 DNA polymerase. One unit of DNA polymerase catalyzes the incorporation of 10 nmol of labeled nucleotide into an acid-insoluble product in 30 min at 30°C. (B) Exonuclease activity was measured using 80 μM alkali-denatured *E. coli* [^{3}H]DNA (64 cpm/pmol/nucleotide) and 13.3 nM T4 DNA polymerase. One unit of exonuclease activity releases 10 nmol of acid soluble product in 30 min at 30°C.


A
D131G
Wild Type
Polymerase Activity (%)
125
100
75
50
25
0
30
35
40
45
50
Temperature (°C)


B
Wild Type
D131G
Exonuclease Activity (%)
125
100
75
50
25
0
30
35
40
45
50
Temperature (°C)

DISCUSSION

Biochemical characterization of functionally distinct mutant T4 DNA polymerases was employed to probe the reaction steps involved in exonucleolytic proofreading. These steps include translocation of the primer-terminus from the polymerase to exonuclease active center, strand separation to produce a partially melted primer-terminus, positioning of the 3'-end of the primer in the exonuclease active center, and finally, hydrolysis of the phosphodiester bond to remove the terminal nucleotide. Although the hydrolysis reaction has been well characterized (Beese and Steitz, 1991), little is known about the reaction steps leading up to hydrolysis. T4 DNA polymerase mutator mutants with amino acid substitutions in the exonuclease domain are presumably deficient in some aspect of proofreading and can, therefore, be used to further probe the details of the proofreading mechanism. Three DNA polymerase mutants with different amino acids substituted for D131 were identified (Reha-Krantz *et al.*, 1986; Reha-Krantz, 1988), which suggests that D131 plays an important role in proofreading. The D131G-DNA polymerase mutant had both temperature sensitive and strong mutator phenotypes, whereas the D131N- and D131S-DNA polymerase mutants were not temperature sensitive and displayed moderate mutator phenotypes.

In order to determine the molecular basis for the mutator phenotypes of the mutant DNA polymerases and, in turn, to gain additional insight into the proofreading mechanism, the D131G-, D131N-, and D131S-DNA polymerases were purified and characterized biochemically. The first step in the characterization of the mutant enzymes was to assay the 5'→3' DNA polymerase and 3'→5' exonuclease activities. Wild type levels of DNA polymerase activity were detected for the D131G-, D131N-, and D131S-DNA polymerases (Table V), which was not unexpected based on the location of D131 in the exonuclease domain. The 3'→5' exonuclease activity, as predicted, was reduced for the mutant enzymes. The D131G-DNA polymerase, which conferred a strong mutator phenotype *in vivo* (Table IV), showed significantly reduced 3'→5' exonuclease activity on both single-

stranded (Fig. 3) and double-stranded (Fig. 4) DNA substrates *in vitro*. A more quantitative measure of exonuclease activity was provided by a sensitive assay which detects the release of fluorescent d2APMP on a millisecond time scale. The excision rates measured for the D131G-DNA polymerase were approximately 200-fold lower than wild type excision rates on both single- and double-stranded DNA substrates (Tables VI and VII). The D131N- and D131S-DNA polymerases had approximately 3- to 10-fold lower excision rates than the wild type enzyme on the double-stranded DNA substrate (Table VI).

The substantially reduced activity of the D131G-DNA polymerase on both single- and double-stranded DNA substrates implies that this mutant enzyme is defective in the chemical step of the hydrolysis reaction. Two possibilities were considered that could explain the substantial decrease in proofreading activity conferred by the D131G amino acid substitution. The 3'→5' exonuclease activity of the D131G-DNA polymerase was in the range of the residual exonuclease activity measured for the D112A+E114A-DNA polymerase (Table VI), which suggested that D131 could be required for hydrolysis as an additional ligand for divalent metal ion coordination. Alternatively, the D131G amino acid substitution could distort the three-dimensional conformation of the exonuclease active center since glycine lacks a side chain and, therefore, may allow the peptide backbone to adopt aberrant conformations.

Several lines of evidence support the second of the two hypotheses. If D131 participates in metal ion coordination, it is expected to be conserved in other DNA polymerases. The T4 DNA polymerase active site residues, D112, E114, D219, and D324 are highly conserved among all proofreading DNA polymerases (Blanco *et al.*, 1992), whereas protein sequence comparisons by Braithwaite and Ito (1993) do not indicate that D131 is a conserved residue. The absence of a residue analogous to D131 in other DNA polymerases argues against a catalytic role for D131.

The temperature sensitivity of the D131G-DNA polymerase mutant *in vivo* also suggests that the glycine substitution affects the conformation of the DNA polymerase.

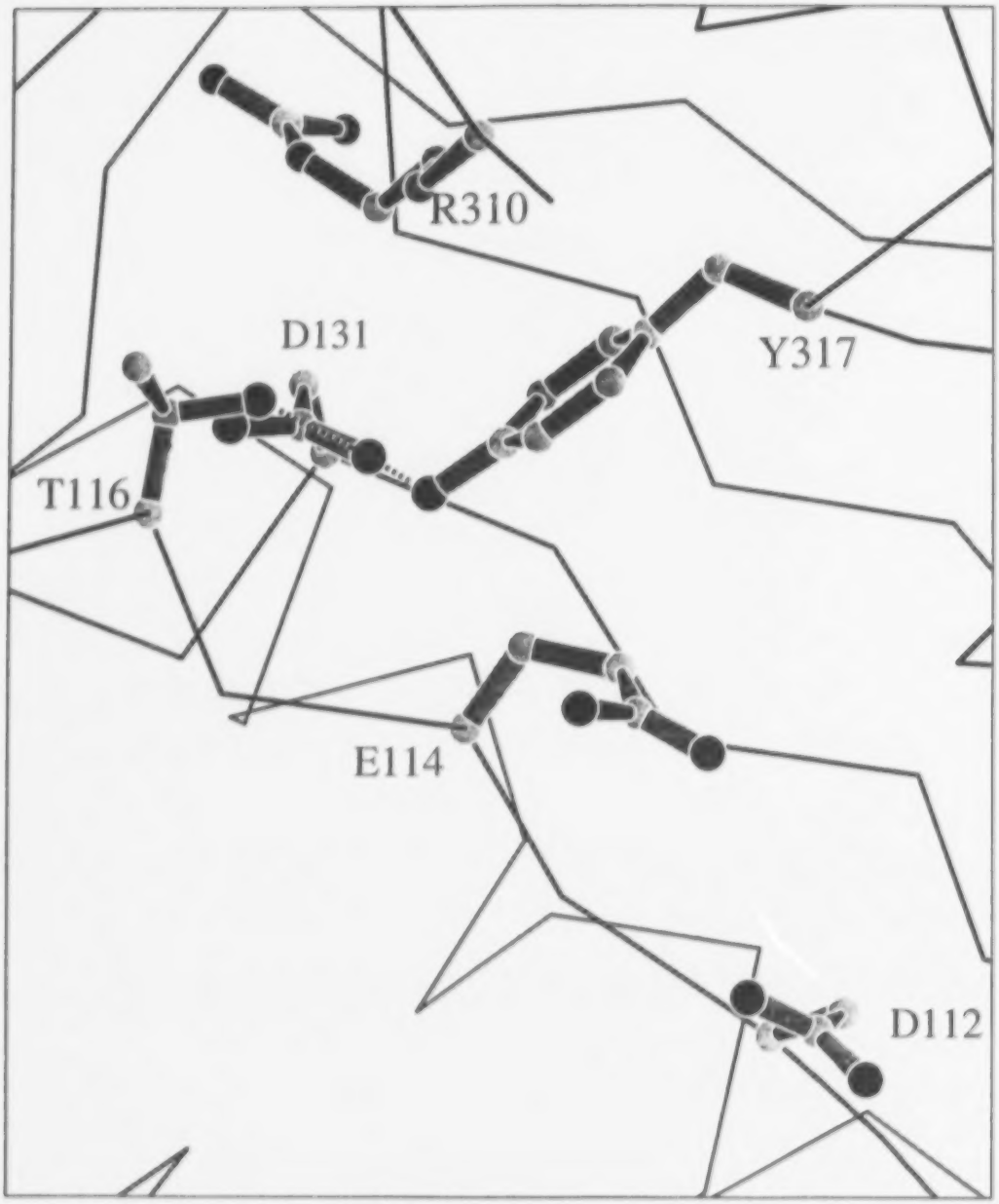
Temperature sensitivity can be the result of decreased protein stability at elevated temperatures or a defect in the proper folding of a protein into its tertiary form at high temperatures (Gordon and King, 1994). The polymerase activity and the residual exonuclease activity of the purified D131G-DNA polymerase were not more sensitive to elevated temperatures than the wild type enzyme (Fig. 9), which suggests that the temperature sensitivity of the D131G-mutant enzyme derives from defective protein folding during synthesis at high temperatures. Although the fully folded D131G-DNA polymerase is temperature stable, temperature sensitivity in folding may indicate an aberrant conformation for the exonuclease active center of the mutant enzyme.

Additional evidence that D131 is likely not a catalytic residue was provided by examining the more conservative D131N and D131S amino acid substitutions for their effects on proofreading. The D131N- and D131S-DNA polymerases had approximately 3- to 10-fold lower exonuclease activity than the wild type DNA polymerase (Table VI). These results are informative when considered in light of the effects of similar amino acid substitutions on metal ion coordination in the Klenow fragment. Asparagine substitutions for active site residues D424 and D501 reduce exonuclease activity by 40,000-fold and 2-fold, respectively, which suggests that asparagine can maintain metal ion coordination at some sites but not at others (Derbyshire *et al.*, 1991). Thus, the question of whether or not D131 is a metal ion ligand cannot be resolved based on the 10-fold reduction in exonuclease activity produced by the D131N amino acid substitution. The similar decrease in exonuclease activity observed for the D131S-DNA polymerase, however, is not consistent with the hypothesis that D131 is a metal ion ligand. Serine substitutions for aspartate are less conservative than asparagine substitutions and, therefore, are not expected to be able to participate in metal ion coordination. Although serine substitutions for Klenow fragment active site residues have not been examined, data are available for alanine substitutions, which are also less conservative than asparagine substitutions. Alanine

substitutions for Klenow fragment residues D355, D424, and D501 reduce exonuclease activity by several thousand-fold (Derbyshire *et al.*, 1991). Therefore, if D131 is a metal ion ligand, the D131S amino acid substitution would be expected to have a more profound effect on exonuclease activity than the D131N substitution. Since the exonuclease activities of the D131N- and D131S-DNA polymerases were comparable, D131 appears to be important for a non-catalytic aspect of the proofreading mechanism.

The recently solved crystal structure of an amino-terminal fragment of the T4 DNA polymerase (Wang *et* al., 1996) also points to a non-catalytic role for D131. The D131 residue is not implicated in coordinating either of the two divalent metal ions required for catalysis; instead, the structural data suggest that a hydrogen bonds exist between D131 and Y317 and between D131 and T116 (Fig. 10) (Jimin Wang and Tom Steitz, personal communication). The Y317 residue is 4 Ångstroms from the catalytic residue, E114, and a water molecule may mediate an indirect interaction between the two residues in the active ternary complex (Jimin Wang and Tom Steitz, personal communication). Such an interaction could be important for maintaining the proper conformation of the exonuclease active center. The hydrogen bond between D131 and Y317 may contribute to the stability of the exonuclease active center by positioning Y317 such that it can interact with E114. Mutational analysis reinforces the biological significance of the D131-Y317 interaction. Genetic selections for mutant DNA polymerases with mutator phenotypes that yielded the D131G-, D131N-, and D131S-mutant DNA polymerases, also identified the Y317C-DNA polymerase (Reha-Krantz *et al.*, 1986; Reha-Krantz, 1988). The Y317C-DNA polymerase, like the D131G-DNA polymerase, confers temperature sensitive and strong mutator phenotypes *in vivo*. The identification of D131G-, D131N-, D131S-, and Y317C-DNA polymerases by genetic selection for mutants with mutator phenotypes suggests that the D131-Y317 interaction observed in crystallographic studies is important for the proofreading reaction.

Figure 10. **Magnified view of the T4 DNA polymerase exonuclease active center.** Residue D131 resides within the exonuclease active center. Proposed hydrogen bonds between D131 and Y317 and between D131 and T116 are illustrated. The Y317 and E114 residues are separated by 4.0 Ångstroms and may interact indirectly via an ordered water molecule.


R310
D131
Y317
T116
E114
D112

Kinetic studies of mutant DNA polymerases with asparagine and serine substitutions for D131 provided additional evidence for the importance of the D131-Y317 interaction. The D131G amino acid substitution was less informative in this respect since it likely results in a gross alteration in the conformation of the exonuclease active center. The D131N- and D131S-DNA polymerases, which displayed significant levels of residual exonuclease activity, appeared to be defective in some aspect of preparing the DNA for the hydrolysis reaction. On the 2AP-T DNA substrates, the rate (k_2) of the slow phase of the biphasic reaction for the D131N- and D131S-DNA polymerases was decreased 10-fold compared to the wild type DNA polymerase (Table VI). A similar reduction in k_2 was measured for the G255S-DNA polymerase (Table X), which is proficient in the hydrolysis of single-stranded DNA. Similarly, the D131N-DNA polymerase showed wild type excision rates on DNA substrates with three terminal mispairs (Table VIII). These results imply that the D131N-DNA polymerase, like the G255S-DNA polymerase, is deficient in some aspect of separating the primer and template strands in preparation for hydrolysis.

The structural data for the amino-terminal fragment of the T4 DNA polymerase provide additional insight into the possible roles of residues G255 and D131 in strand separation. The G255 residue is located in a loop structure of the T4 DNA polymerase which is proposed to assist in strand separation by forming a wedge between the primer and template strands (Marquez and Reha-Krantz, 1996). The D131 residue is not found within the loop structure, but resides deep within the exonuclease active center, which suggests that D131 affects the strand separation process in a different manner. From the excision experiments with the D131N-DNA polymerase on DNA substrates with increasing pre-formed strand separation, exonuclease activity of the mutant enzyme remained lower than wild type until three terminal mispairs were provided. These results suggest that the D131 residue may be involved in the conversion of a primer-terminus with two terminal unpaired bases to a primer-terminus with three terminal unpaired bases. Based on the location of D131 in the exonuclease active center, the role of this residue in strand

separation may be to interact directly (or indirectly through its interaction with other residues, such as Y317) with the 3'-terminal nucleotide of the primer strand to stably position it within the exonuclease active center.

The reduced efficiency of strand separation observed for the D131N-DNA polymerase is expected to increase the kinetic barrier to proofreading by increasing the probability of extension relative to excision. In order to simulate the competition between extension and excision *in vitro*, the excision of d2APMP was examined in the presence of dNTPs. Extension of the terminal 2AP-T base pair could only be observed if the primer-terminus is capable of binding productively in the polymerase active center. The formation of an Enzyme^{pol}:2AP-T DNA complex has been proposed to account for the slow phase of the biphasic excision reaction, since DNA bound in the polymerase active center would have to be transferred to the exonuclease active center prior to hydrolysis. This prediction was confirmed by extension:excision competition experiments, since extension of the 2AP-T base pair was catalyzed by the wild type, D131S-, D131N-, and D131G-DNA polymerases.

The wild type and mutant DNA polymerases differed, however, in their ability to extend a terminal 2AP-T base pair. In the presence of dNTPs, the D131S- and D131N-DNA polymerases extended the terminal 2AP-T base pair more frequently than the wild type enzyme, while only extension, to the exclusion of excision, was observed for the D131G-DNA polymerase (Fig. 8). These results suggest that the D131 mutants are defective in some aspect of converting a mispaired primer-terminus initially bound in the polymerase active center into a single-stranded primer-terminus bound within the exonuclease active center. The increased tendency of the D131G-, D131N-, and D131S-DNA polymerases, compared to the wild type enzyme, to extend the "mispaired" 2AP-T primer-terminus, caused by their decreased ability to proofread, is consistent with the increased incidence of spontaneous mutations produced by the mutant DNA polymerases *in vivo*.

Summary and Future Directions

The role of D131 in exonucleolytic proofreading was investigated by characterizing the D131G-, D131N-, and D131S-mutant DNA polymerases *in vivo* and *in vitro*. All three mutant DNA polymerases conferred mutator phenotypes *in vivo* and displayed reduced 3'→5' exonuclease activity *in vitro*. The results discussed here, combined with recent crystallographic data (Wang *et al.*,1996) suggest that a hydrogen bond between D131 and Y317 is important in maintaining the structural conformation of the exonuclease active center required for efficient proofreading. The alteration in protein conformation conferred by disrupting the D131-Y317 interaction is proposed to affect the relative partitioning of the primer-terminus between the polymerase and exonuclease active centers such that a mispaired primer-terminus is less likely to be proofread. The reduced proofreading activity accounts for the increased frequency of spontaneous mutations produced by the mutant DNA polymerases *in vivo*.

To further investigate the importance of the D131-Y317 interaction in proofreading, additional mutant enzymes could be constructed and characterized. An alanine substitution for D131 would be less disruptive than the glycine substitution to overall protein conformation, but the small, hydrophobic alanine residue would prevent hydrogen bonding to Y317. Thus, the D131A-DNA polymerase is predicted to have very low exonuclease activity if the hydrogen bonds with D131 are essential to the proofreading reaction. Similarly, the T116A and Y317F amino acid substitutions would retain structure but prevent hydrogen bonding, such that the mutant DNA polymerases are expected to have decreased exonuclease activity if the proposed hydrogen bonds to D131 are indeed important for stabilizing the exonuclease active center.

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